EMERGING EPIGENETICS IN CANCER CHEMOPREVENTION BY DIETARY PHYTOCHEMICALS

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ABSTRACT OF THE DISSERTATION

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Cancer chemoprevention is defined as the strategy to block or slow the onset of premalignant tumors and using relatively nontoxic chemical substance. Recently, accumulating experimental evidence has suggested that epigenetic alterations are involved in cancer development. The scope of epigenetics lies on the molecular interface between genetics and environmental factors; external factors switch genes on and off by influencing how cells read the genes. DNA methylation, histone covalent modification and remodeling, miRNA-mediated gene silencing represent the major mechanisms that play important role in epigenetic control of gene expression. This thesis focused on elucidating the epigenetic mechanisms in cancer prevention by dietary phytochemicals. Nrf2 is a master regulator of the antioxidant response and xenobiotic metabolism through the regulation of a wide range of antioxidant and phase II detoxification genes. The cellular protective role of Nrf2 points its potential as a primary target in chemoprevetion. Our group has reported that within tumor development in the transgenic adenocarcinoma of mouse prostate (TRAMP) model, there is a progressive loss of expression of Nrf2 and its downstream target genes, which is

associated CpG hypermethylation in the promoter region. Using TRAMP C1 cells, we demonstrated that sulforaphane is a potent demethylation agent and it restores the epigenetically silenced Nrf2 gene through DNA demethylation. JB6 cells are derived from normal skin epidermis. We found that hypermethylation of Nrf2 promoter also exists in the transformation sensitive JB6 P+ cell line. The epigenetic reactivation of the Nrf2 signaling pathway by Tanshinone IIA could potentially contribute to the attenuation of JB6 P+ cellular transformation under the challenge of TPA, a tumor promoter. On the other hand, histone modification, in particular acetylation of H3K27 residue, is implicated in the transcription activation of pro-survival, pro-proliferative, and pro-inflammatory genes following TPA treatment. Bromodomain and extraterminal domain (BET) proteins function as epigenetic reader that recognizes acetylated histone tails and recruits the transcription machinery. Our study revealed that a small molecule BET inhibitor JQ-1 exerts potent anti-cancer and anti-inflammatory effects by interfering with the core transcriptional program of neoplastic transformation. Last but not least, altered levels of miRNA have been linked to tumor malignancy due to their ability to regulate functional gene expression in carcinogenesis. Using oligonucleotide microarray approach we identified the most affected miRNAs in LNCaP cells. Then we further assessed one potential target of PEITC - miR-194 in prostate cancer cell invasiveness. Collectively, dietary phytochemicals could modulate cellular epigenetic events that in part contribute to the cancer preventive effects. Given that oxidative stress and inflammation reaction are important (micro) environmental factors in malignancy transformation, understanding the role of redox and inflammatory signaling in epigenetic regulation could bring novel insights in cancer prevention.

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DEDICATION

To Wenyi, and My parents

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1.1 Introduction

Over the last decades, there have been incredible advances in cancer detection, prevention, and treatment. In the United States, the cancer statistics from National Cancer Institute (NCI) have indicated that the overall cancer death rate has declined steadily since the early 1990s. Clearly, progress is being made in the fighting against the disease, but still much work remains. Until now, cancer has been a heavy social and economic burden; it is the second leading causes of death while expenditures for cancer care is increasing. In addition, mortalities for a few cancers have been staying stably or even increased. Therefore, tremendous efforts have been continuously made in cancer research.

Cancer begins in normal cells in the body. Malignant transformation is a dynamic process which involves accumulation of alterations on the genes controlling cell differentiation, proliferation, survival and apoptosis (1) (Figure 1.1). Despite the highly complex and heterogeneous nature of the disease, cancer development can be generally characterized by three stages – namely initiation, promotion and progression (2). Cancer initiation is often a rapid event, derived from mutation in one or more genes controlling

[1] Part of this chapter has been submitted to an international journal.
[2] **Key Words**: chemoprevention, phase II enzymes, Nrf2, epigenetics, oxidative stress, inflammation, DNA methylation, histone modification
[3] **Abbreviations**: DNMT, DNA methyltransferase; HDAC, histone deacetylase; GST, glutathione S-transferases; UGT, UDP-glucuronosyl transferases; NQO, NAD(P)H quinine oxidoreductase; ARE, antioxidant response element; Nrf2, nuclear factor-erythroid 2 (NF-E2)-related factor 2; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; ITC, isothiocyanate; PEITC, phenyl isothiocyanate; SFN, sulforaphane.

key regulatory pathways of the cell. Triggered by chemical carcinogens, virus infection, radiation etc., failure of the DNA repair mechanisms is irreversible but heritable in tumor initiating cells. Tumor promotion is a possible prolonged process that enhances the effect of the genotoxic initiating agent by establishing clones of initiated cells and is considered reversible. In the progression phase, the complex cellular interactions between further mutations from genetic instability, recruitment of immune inflammatory cells, and angiogenesis factors etc. lead to tumor invasion and metastasis.

Cancer can be triggered by many factors, such as genetics, diet, environment, and occupation. According to the scheme mentioned above, there could be some possible window to intervene or slow tumor development. Indeed, inhibition of each stage of carcinogenesis by administering chemical agents was documented as early as the 1960s (3). In 1976, Dr. Michael B. Sporn first introduced the conception of "chemoprevention" in his research on the prevention of malignancy development by retinoids (a group of synthetic vitamin A analogs) (4). The basic idea of cancer chemoprevention is thus described as a strategy to block or slow the onset of premalignant tumors using relative nontoxic natural phytochemicals or pharmaceutical agents, both refer to chemopreventive agents. Based on the mode of action chemopreventive agents were classified into two categories: (1) blocking agents that prevent the initiation stage by inactivating carcinogens or enhancing detoxifying enzyme activities; (2) suppressing agents that inhibit cell growth or induce apoptosis thereby intervening the malignant transformation of initiated cells at either the promotion or the progression phase (Figure 1.2).

Dietary and medicinal plants are rich resources of phytochemicals which have played an important role in the cancer prevention practice (5). Epidemiological evidences

have suggested the correlation between incidence of cancer and consumption of certain type of fruits and vegetables (6). Dietary phytochemicals such as curcumin from turmeric, flavonoids from soybeans, isothiocyanates from cruciferous vegetables, and polyphenol from green teas etc. have been shown to be effective in protecting against different types of tumor development (7-10). Animal carcinogenesis models and cancer cell lines have provided valuable insights in evaluating the chemopreventive potential of phytochemicals, as well as elucidating the mechanisms of cancer prevention effects. According to current knowledge, rather than elicit unique signal transduction pathways, most chemopreventive agents could trigger some common cellular events, such as increasing gene expression of detoxifying/antioxidant enzymes, modulating immunity, inhibiting cell cycle progression and cell proliferation, and inducing differentiation and apoptosis etc (11). In general, antioxidant and anti-inflammatory effects are tightly related to genomic stability, cell growth, proliferation and apoptosis, which remain to be important primary targets in chemoprevention (12). Recently, emerging evidences have demonstrated that epigenetic alterations are involved in different stages of cancer. The epigenetic mechanisms underlying the chemopreventive effects of phytochemicals, as well as the influence of redox signaling and inflammatory response on the epigenetic scope have gained much attention, which are also our major study interests.

1.2 Targeting epigenetic regulations in cancer

The term epigenetics refers to the study of dynamic alterations in the gene transcriptional potential without changes in the primary DNA sequence. The scope of epigenetics lies on the molecular interface between genetics and environmental factors; external factors switch genes on and off by influencing how cells read the genes. DNA

methylation, histone covalent modification and remodeling, miRNA-mediated gene silencing represent the major mechanisms that play important role in epigenetic control of gene expression, such as tissue differentiation, genomic imprinting, and X-chromosome inactivation etc. Cellular gene expression is tightly and spatiotemporally controlled by chromatin structure. The interplay of DNA methylation, histone modifications, transcription factors/co-factors, and DNA binding proteins determines the status of gene transcription, which is essential in maintaining cellular differentiation and homeostasis states. During cancer development, long-lasting effects such as aging process and environmental interactions introduce alterations in epigenome, which determine the susceptibility of cells to malignant transformation (13, 14). In addition, studies on carcinogenesis associated epigenetic abnormalities suggest that epigenetic disruption may interact with genetic dysregulation to exacerbate disease progression (15). However, unlike genetic mutations, aberrant epigenetic switches tend to be acquired in a gradual process and are potentially reversible. Therefore, targeting epigenetics has been considered as a novel strategy in cancer prevention and therapy.

1.2.1 DNA methylation

DNA methylation is the first covalent DNA modification identified in mammalian cells (16), which is the process of adding a methyl group on the 5’ position of cytosine residues (5mC) within CpG dinucleotides. These CpG dinucleotides are often rich in the regulatory regions in 5’ ends of many genes, known as CpG islands. Indeed, CpG islands are present in over 70% mammalian gene promoters. DNA methylation plays critical role in maintaining genome stability, regulating chromatin architecture, and controlling gene transcription activity. Empirically, human neoplasia may be characterized as an imbalance

of DNA methylation, involving global hypomethylation and regional hypermethylation (17). Loss of widespread DNA methylation has been observed as a major characteristic in different types of cancer, which may lead to genomic instability due to inappropriate activation of retrotransposons and other silent genomic regions (18). On the other hand, aberrant CpG island hypermethylation is associated with inappropriate gene silencing. This epigenetic inactivation involves key molecules in DNA repair, cell cycle, apoptosis, cell adherence, and detoxification etc., which appears to be important in driving carcinogenesis (19). In addition, abnormal hypermethylation is associated with genetic lesions due to the profound effects on genomic stability (20).

DNA methylation is facilitated by a family of DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, and DNMT3B. Under certain physiological or pathological conditions, DNMT3A and DNMT3B are responsible for establishing *de novo* methylation patterns, which are then maintained by DNMT1 during DNA replication. A cytidine analog, 5-azacytidine (AZA, Vidaza ®), was the first DNMT inhibitor approved by the U.S. Food and Drug Administration (FDA) in 2004 for patients with myelodysplastic syndrome (MDS). Mechanistic study indicated that AZA can form an irreversible covalent complex with DNMT1 that further leads to proteasome-mediated degradation (21). AZA, as well as another DNMT inhibitor 5-aza-2'-deoxycytidine (DAC, Dacogen ®), have shown clear clinical benefits with minimal toxicity in trials for MDS and acute myeloid leukemia (AML). In addition, the clinical outcome of DAC treatment in MDS patients appeared to be highly correlate with DNA methylation status in a number of genes (22).

Additionally, the presence of 5-hydroxymethylcytosine (5hmC) lead to the recognition of the role of ten-eleven translocation (TET) family in regulating DNA

methylation (23). By converting 5mC into 5hmC, TET proteins erases or alters cellular DNA methylation through both active and passive DNA demethylation (24). Consistent with the notion of 5mC and 5hmC, TET proteins are likely to play a role in in regulating gene expression and maintaining cellular identity. It has been reported that Tet2 deficient mice have a chronic myelomonocytic leukemia (CMML) phenotype (25). Accordingly, TET2 mutations have been observed in numerous human hematological malignancies (26, 27). However, the clinical implications of TET2 mutations are largely inconclusive so far; no TET protein inhibitor has been tested for cancer treatment in clinical.

1.2.2 Histone modifications

Eukaryotic genome is organized in a hierarchical structure known as chromatin, which is comprised of DNA, histones, and DNA-binding proteins. Chromatin organization could affiliate the regulation of gene expression by allowing/restricting the access of different DNA binding proteins or protein complexes to the genetic material. Methylated DNA can initiate recruitment of the methylation-dependent DNA binding domain (MBD) family, including methyl CpG-binding protein 2 (MeCP2) and MBD1-4 (28). These MBD proteins, as well as DNMT1, could further recruit histone deacetylase (HDAC) to the target sites, which draws another level of epigenetic mechanisms - histone modifications. Histones are not only DNA-packaging proteins. Chromatin remodeling is triggered by various post-translational modifications on histone tails such as lysine acetylation, arginine and lysine methylation, and serine phosphorylation, which could affect gene transcription and DNA repair. It has been proposed that the type and sequence of histone modifications enciphers a ‘histone code’ that extends the cellular information potential of genetic code (29).

Histone lysine acetylation is among the best studied histone modifications (30). The negative charge conferred by the acetyl moieties leads to open up of the chromatin, which is generally associated with transcriptional activation. Acetylation is a highly dynamic balance regulated by the competition of two enzyme families, the histone lysine acetyltransferases (HATs) and the HDACs. HATs are classified into three subtypes, including the GCN5 N-acetyltransferase (GNAT), the MOZ/YBF2/SAS2/TIP60 (MYST), and the p300/CBP families. The importance of HATs in cancer was immediately apparent since they were identified (31). Although chromosomal translocation and mutation associated excessive HAT activities are found in a broad range of hematological and solid malignancies (32, 33), developing specific inhibitors against HATs has proved to be challenging (34). One primary reason is the limited structural homology between the different families of HATs. In addition, non-histone acetylation mediated by these lysine acetyltransferases could have complex influence on oncogenes and tumor suppressors such as MYC, p53, and PTEN etc. However, studies suggest that HAT activities could be inhibited to some extend by phytochemicals such as curcumin, anacardic acid, garcinol and their derivatives.

Histone deacetylases (HDACs) remove the acetyl groups from lysine residues that is usually associated with chromatin compaction. Four classes of HDACs have been identified in humans, among which Class I, II and IV are zinc-dependent HDACs, while class III HDACs (as known as sirtuins) are NAD+-dependent. Despite the challenges in dissecting the effects of HDACs on histone and non-histone proteins (35), overexpression and altered activities of various HDACs appear to be involved in numerous cancers (36). Inhibition of HDACs is able to reactivate cell cycle regulatory protein, inhibit cell growth,

facilitate differentiation, and induce apoptosis in malignant cells (37). To date, two non-selective HDAC inhibitors, Vorinostat and Romidepsin, have been granted FDA approval for treatment of cutaneous T cell lymphoma. Selective HDAC inhibitors with distinct spectrums are currently under different stages of clinical trials target several indications.

Histones methylation occurs on the side chains of arginine, lysine, and histidine residues, among which lysine modifications are best characterized so far. Unlike acetylation, the functional consequence of the histone methylation is site dependent; methylation of the fourth lysine residue on histone 3 (usually present as H3K4me) is closely linked to transcriptional activation, whereas H3K9me3, H3K27me3, H4K20me are markers for transcriptional repression (38). Histone methylation status is controlled by two group of enzymes carrying opposite functions, histone lysine methyltransferases (HMTs) and histone demethylases (HDMs). In contrast to the HATs and HDACs, the HMTs and HDMs tend to be highly site specific enzymes that target certain lysine residues. The fact that lysine residue could be unmethylated, monomethylated, dimethylated, or trimethylated further adds to the complexity of enzymes responsible for histone methylations. Most HMTs contain a conserved element, namely Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain, which possesses methyltransferase activity. HDM proteins also have a variety of regulatory domains. According to enzymatic protagonists HDMs could be classified into lysine-specific demethylases (LSDs) and Jumonji demethylases (JMJCs). Currently, development of drug targeting HMTs and HDMs are still in its infancy. A small number of candidates such as DOT1L, EZH2, and LSD1 inhibitors have entered clinical phase (39-41).

While enzymes adding covalent modifications on histone tails are considered as "writers" and "erasers" of histone code, there are groups of proteins function as "readers", e.g. bromodomain and extraterminal (BET) proteins and plant homeodomain (PHD) finger proteins etc., that specifically recognize those chromatin markers and regulate transcription machineries in response to upstream signaling (42). Mutations in these epigenetic reader genes may abrogate the chromatin-reading capacity, which play an influential role in a variety of cancers (43). Targeting these chromatin reader may offer a unique opportunity to tailor therapies, especially in some cases direct target inhibition approach is not available. For instance, inhibition of MYC associated oncogenic gene transcription by BET inhibitor JQ1 has been proven to be effective (44). Investigations on novel chemical entities inhibiting the epigenetic readers are ongoing for developing orthogonal and synergistic cancer interventions.

1.3 Nrf2, redox signaling and epigenetic modifications

Cellular defensive systems are essential in maintaining homeostasis towards environmental or intracellular challenges, such as oxidative stress. Phase II enzymes (e.g. NAD(P)H: quinone oxidoreductase, NQO-1; glutathione S-transferases, GST; UDP-glucuronosyl transferases, UGT; among others) are well-known as detoxifying enzymes that conjugate endogenous polar molecules to the phase I metabolites , thereby facilitating xenobiotics (including carcinogens) elimination and excretion (45). Chemopreventive effects of phytochemicals have largely been attributed to their ability of enhancing the capacity of cellular defensive system by inducing phase II detoxifying and antioxidant enzymes (46).

The promoter region of most phase II and antioxidant genes share a common sequence called antioxidant response element (ARE) (47). The transcription activities of these ARE-containing genes are generally controlled by nuclear factor (erythroid-derived 2)-like 2 (NFE2L2, Nrf2), a helix-loop-helix basic leucine zipper (bZIP) transcription factor. In unstimulated cells, Kelch-like ECH-associated protein 1 (Keap1) anchors Nrf2 in cytosol, bridging the Nrf2-Keap1 complex to Cul-3-dependent ubiquitination then undergoing proteosomal degradation (48). The activation of Nrf2 pathway involves disassociation of Nrf2 from Keap1 binding and nucleic translocation of Nrf2. In nucleus, Nrf2 forms heterodimers with Mafs, subsequently binds to the cis-acting ARE/electrophile response element (EpRE) in the promoters, and enhances the transcription activity of those phase II/antioxidant genes (49). Thus, the Nrf2 pathway has been proposed as a primary cellular target for chemoprevention.

As mentioned above, it is not surprising that gene expressions in Keap1-Nrf2 signaling can be regulated by epigenetic mechanisms (reviewed by (50)). On the other hand, the Nrf2 pathway controls the activation of many key molecules in regulating cellular redox status, which could have certain influence on the epigenetic landmarks. For example, it has been reported that under oxidative stress methionine sulfoxide (from oxidative damage to methionine residues in proteins) could react with ·OH to generate methyl free radicals that result in non-enzymatic methyl-adduct on cytosine (51). On the other hand, the catalytic activities of those epigenetic enzymes may be affected by cellular redox status. DMNTs and HMTs utilize a similar reaction mechanism in which S-adenosyl methionine (SAM) donates a methyl group to form methyl-DNA or histone adducts and the byproduct S-adenosyl homocysteine (SAH). Oxidative stress result in a reduced GSH/GSSG ratio,

which in turn stimulates glutathione synthesis. Less available SAM results from the competition between cellular glutathione and SAM generation may further cause methyltransferase disruption. JMJC, LSD and TET proteins all belong to the superfamily of 2- oxoglutarate (2-OG)- and Fe(II)-dependent dioxygenases (52). Cells have strict regulatory controls of labile iron pool that can be disrupted in the presence of pro-oxidants. It has been demonstrated that HDAC2 activity could be reduced by hydrogen peroxide (H_2O_2), a common reactive oxygen intermediate in cells. Tyrosine nitration of HDAC2 is associated with increased IL-8 expression following an increase in acetylated H4 at the promoter (53).

Although the precise mechanism remains to be elucidated, in general it appears that redox perturbations can have significantly broader consequences on enzymatic activities of epigenetic modulators. When cells are experiencing prolonged oxidative stress, multiple processes are possibly taking place that ultimately lead to epigenetic abnormalities. Balancing reactive oxygen species (ROS) is likely to be important in respect of the epigenomic landscape.

1.4 inflammation and epigenetics

The phenomenon that tumors are frequently infiltrated with immune cells was observed as early as in the 19th century (54). Chronic inflammatory disorders have been long considered as cancer susceptibility factors whereas approximately 20% cancer deaths are associated with chronic infection and inflammation (55). In physiological condition, inflammation is a necessary and beneficial response to tissue injury and pathogenic agents. However, aberrantly prolonged inflammation can promote malignant cell transformation by direct or indirect interactions with tumor cells in the microenvironment (56). Cytokines,

chemokines, and growth factors secreted from infiltrating immune cells can promote cell survival, proliferaction and inhibit apoptosis, as well as extracellular matrix-modifying enzymes and pro-angiogenic factors that promote epithelial–mesenchymal transition (EMT), invasion, and metastasis (57). Recent studies also suggest that the pro-tumorigenic functions of inflammatory signaling are partially mediated by epigenetic mechanisms (58).

Along with being epigenetically regulated in malignancies, inflammatory genes also play an important role in the modulation of cellular epigenetic events such as DNA methylation. The pro-inflammatory cytokine interleukin-1 beta (IL-1β) enhances DNMT activity via nitric oxide production (59), which may further result in promoter methylation and reduced transcriptional activity of the CDH1 gene encoding E-cadherin (60). In addition, exposure to another pro-inflammatory cytokine interleukin 6 (IL-6) was found to transcriptionally induce and stabilize the DNMT1 protein, which may contribute to epigenetic silencing of genes associated with tumor suppression, adhesion, and apoptosis resistance (61). Interestingly, elevated lysine acetylation of IL-6 downstream effector, signal transducer and activator of transcription 3 (STAT3), has been commonly observed in cancer and is considered to interact with DNMT1 to facilitate aberrant DNA methylation of tumor suppressor genes (62). Transforming growth factor beta (TGF-β) is a key molecule in cell migration, which induces expression and activity of DNMT1, DNMT3A, and DNMT3B to cause global changes in DNA methylation in EMT (63). Thus, cytokine signaling associated epigenetic switches could participate in tumor initiation and progression. In a mouse colitis model induced by dextran sulfate sodium (DSS), increasing DNA methylation of FOSB, HOXA5, and KRT7 genes were identified in colon epithelial cells exposed to colitis before colon cancers developed (64). In contrast, exposure of

cultured colonic epithelial cells to DSS did not alter DNA methylation patterns in above genes. In addition, these changes in DNA methylation were at the same levels in mice models that lack function T- or B-cells. Therefore, infiltrating macrophages and associated inflammation response triggered by the DSS treatment might be responsible for the methylation alterations. Last but not least, decreased levels of global 5hmC were found in chondrocytes under IL-1β and tumor necrosis factor alpha (TNF-α) treatment, indicating that inflammation may also modulates DNA methylation via 5hmC (65).

Inflammation can also affect epigenetic regulation in scope of histone modifications. As discussed in previous section, epigenetic control of certain genes could be in a bivalent state, wherein the gene regulatory regions carry both repressive (i.e., H3K27me3) and active histone codes (i.e., H3K4me3) simultaneously. These bivalent domains tend to control genes expressed at low levels, but poised for activation (66). ZEB1 gene has been shown to adopt such bivalent state that enable the switching of non-CSC to CSC status in breast cancer cells (67). Stimulation with TGF-β reduced the presence of repressive H3K27me3 while enhanced the active H3K4me3 mark at the ZEB1 promoter. This switch on chromatin resulted in enhanced expression of ZEB1 and consequent EMT and conversion from non-CSCs to CSCs. Furthermore, although DNA methylation remained unchanged, reprogramming of specific chromatin domains across the genome was observed during TGF-β induced EMT (68); characterized by a global reduction of heterochromatin mark H3K9Me2 and an increase of the euchromatin mark H3K4Me3 and H3K36Me3. On the other hand, inflammation signal may also reshape the epigenome by targeting a certain histone residue. HOTAIR is a long non-coding RNA functions as a scaffold for coordination of PcG complex mediated H3K27 methylation (69). The

expression of HOTAIR is nearly two-thousand higher in breast cancer metastases compared to that of normal breast epithelium. Overexpression of HOTAIR resulted in genome wide retargeting of PcG complex and consequent deregulation of H3K27me3 marks in a variety of genes, thereby increasing invasiveness and metastatic potential. Recently, it has been shown that the activated IL6/STAT3 signaling could elevate the expression of HOTAIR during malignant transformation of human bronchial epithelial cells induced by cigarette smoke extract (70), suggesting that HOTAIR could be a mediator between inflammation and epigenetic reprogramming. Taken together, inflammation can induce various epigenetic alterations that range from specific gene regulation to genome-wide reprogramming, which may culminate in epigenetic switches that promote cancer development.

1.5 Integrate epigenetics in cancer prevention using isothiocyanates (ITCs)

The widespread availability and rather safe toxicity profile of the healthy benefitting phytochemicals rise to continuous enthusiasm on evaluating their potential in prevention and therapy in many diseases including cancer. In specific, numerous epidemiological and pharmacological studies suggest a correlation between consumption of cruciferous vegetables and reduced cancer risk in human (71, 72). Over 200 naturally-occurring glucosinolates are found rich in cruciferous vegetables (73), which consist of a β-D-thioglucose group, a sulfonated oxime group, and a side chain derived from methionine, phenylalanie, tryptophane, or branch-chained amino acids (74). Interestingly, the chemopreventive effects are mostly attributed to the isothiocyanate (ITC)-containing compounds rather than their glucosinolate precursors. ITCs, converted by myrosinase

mediated hydrolysis from glucosinolate, are characterized by the sulfur containing N=C=S functional group with a wide structural diversity. Ally isothiocyanate (AITC) from cabbage, mustard, and horseradish; benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) from watercress and garden cress; and sulforaphane (SFN) from broccoli, cauliflower, and brassicas have been mostly studied against a variety of human malignancies (75). In cell culture models, micromolar concentrations of ITCs have shown potent anti-cancer effect through different mechanisms *in vitro* (76, 77). Several pharmacokinetic studies provided evidence that the concentration range is achievable *in vivo*. For example, oral administration of 50μmol SFN in rats would result in a peak plasma concentration of 20μM at 4 h (78). Plasma concentration could reach to 9.2 and 42.1μM after an oral dose of 10 and 100μmol/kg PEITC in rats (79). In a chemopreventive study using the ApcMin/+ mouse model, SFN inhibited adenoma formation with a steady-state concentration of 3-13nmol/g (roughly equivalent to 3-10μM) in the gastrointestinal tract (80). While anti-oxidant and anti-inflammatory effects of ITCs have been long considered as primary targets for chemoprevention, epigenetic mechanism are emerging (Figure 1.3).

1.5.1 ITCs induce phase II enzymes through Nrf2/ARE pathway

The chemopreventive effect of ITCs is considered to be associated with their ability to induce the expression of phase II enzymes. It has been extensively documented that SFN exerts potent activation of phase II/antioxidative gene expression in both *in vitro* and *in vivo* studies (81). In rats, 40 μmol/kg/day SFN treatment was found to increase GST and NQO1 activities in the duodenum, forestomach, and bladder tissues (82). In hepatocytes, SFN induced UGT1A1 and GSTA1 mRNA expression and protected cells against the 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP, commonly found in cooked meat

and considered as risk factors for cancer)-DNA adduct formation (83). Similarly, PEITC was found to induce hepatic phase II enzymes, resulting in decreased PhIP-DNA adduct levels in rat tissues (84). In mice, 12 h after an oral dose of PEITC, upregulation of several GST isozymes in the liver was identified using a microarray approach (85). Markedly, a number of studies on ITCs suggest that the induction of phase II/antioxidant enzymes is NF-E2-related factor-2 (Nrf2) dependent (85-87).

Mechanistic studies demonstrated that ITCs could activate Nrf2 pathway by modifying the Nrf2-Keap1 interactions. In specific, using a liquid-tandem mass spectrometry approach, Hong et al. provided evidence that SFN could directly react with thiol groups of Keap1. The formation of SFN-Keap1 thionoacyl adducts releases Nrf2 from the Nrf2-Keap1-Cul3 degradation complex; this stabilization of cellular Nrf2 consequently results in Nrf2 nuclear translocation and activation (88). On the other hand, PEITC induces the Nrf2/ARE signal through a different mechanism mediated by mitogen-activated protein kinases (MAPKs). It has been reported that PEITC induced ARE activity was attenuated by c-Jun N-terminal kinase-1 (JNK1) and extracellular signal-regulated kinase (ERK) inhibitors (87). In the same study, *in vitro* kinase assays showed that JNK1 and ERK2 could directly phosphorylate Nrf2 protein. Collectively, PEITC increased the phosphorylation of ERK1/2 and JNK1/2 in cells, which, in turn, caused phosphorylation of Nrf2 and subsequent release from Keap1 binding, and resulted in translocation activation of the Nrf2/ARE pathway. To note, Nrf2-deficient mice have shown increased susceptibility in carcinogenesis models and less effective towards preventive treatment (89, 90). Therefore, transcriptional induction of Nrf2/ARE mediated phase II enzymes is considered as an important mechanism for the chemopreventive effects of ITCs.

1.5.2 Anti-Inflammatory effects of ITCs

Inactivation of NFκB pathway by ITCs could be another important mechanism that contributes to their anti-cancer activity. Experimental evidence suggests that ITCs could stabilize IκB by inhibiting its phosphorylation and degradation, resulting in attenuated nuclear translocation of p65 (a subunit of NFκB) and NFκB activation. In PC-3 cells, both SFN (20 and 30 μM) and PEITC (5 and 7.5 μM) strongly inhibited nuclear translocation of p65, with the concomitant decreased expression of NFκB regulated genes such as Bcl-XL, cylcin D1, and vascular endothelial growth factor (VEGF) (91). Correspondingly, PEITC and SFN were found to inhibit lipopolysaccharide (LPS)-induced NFκB luciferase activity in HT-29 cells, which was also mediated through inhibition of IκKβ phosphorylation (92). In addition, SFN was proposed to be able to interact with glutathione and other redox regulators like Ref-1 or thioredoxin, which in turn indirectly impairs the NFκB-DNA binding ability (93). Another study by Heiss E et al suggested that SFN directly interacts with Cys residues of NFκB subunits by forming dithiocarbamate, which results in decreased DNA binding abilities (94). Collectively, these findings indicate that redox modulation and thiol reactivity play certain roles in regulating NFκB-dependent transcription by SFN. Interestingly, studies on the cross-talk between Nrf2 and NFκB signaling have shown that Nrf2 downstream target may inhibit of NFκB nuclear translocation (95, 96). Accordingly, pre-treatment of SFN (25 mg/kg per day) mitigated dextran sodium sulphate (DSS)-induced acute colitis *in vivo*, while increased expression of Nrf2-dependent genes and reduced expression of inflammatory was observed in the colon tissues (97). Similarly, SFN restored the number of sunburn cells back to the basal level in Nrf2 WT but not Nrf2 KO mice after UV irradiation. The inflammatory markers were

lower in SFN treated Nrf2 WT tissues compared to Nrf2 KO tissues (98). These results suggest that activation of Nrf2 by SFN could at least in part contribute to the suppression of pro-inflammatory signaling pathways.

1.5.3 Epigenetic modulation targeted by ITCs

Diet has been considered as an important environmental factor that could affect epigenetic events. In the context of chemoprevention, there is a growing interest in evaluating the potential of dietary phytochemicals that blocks or reverses the epigenetic abnormity in cancer development. In specific of ITCs, Wong et al. reported the effects of SFN on promoter DNA methylation profile in prostate epithelial cells (PrEC), androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cells (99). SFN treatment was found to decrease the DNMT levels in all the tested cell lines. Although SFN showed complex effects on genome-wide DNA methylation patterns among normal prostate epithelial and prostate cancer cells, the genes of altered methylation status were functionally similar within a single cell line (e.g. cell migration, cell adhesion etc). In various *in vitro* and *in vivo* studies, SFN or PEITC treatments appeared to downregulate DNMTs activity, thereby resulting in promoter demethylation of epigenetically silenced genes, with the concomitant change of gene expressions (reviewed by (100, 101)). Interestingly, DNA demethylation in promoter region is often found to be associated with local relax histone structure, although the precise mechanism remains to be elucidated. For example, in mouse epidermal JB6 (JB6 P+) cells, SFN demethylated the CpGs in Nrf2 promoter region via inhibition of DNMT activities, as well as HDACs. This change in methylation pattern is associated with increased Nrf2 level and a phenotype that more

resistant to 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced neoplastic transformation (102).

On the other hand, HDACs are often upregulated in cancers therefore HDAC inhibition is considered as an important strategy in cancer prevention and therapy. Molecular docking indicated that metabolite of SFN and several structural related ITCs could directly interact with HDAC catalytic core to inhibit the enzyme activity (103). In a clinical study, a single dose of 68 g broccoli sprouts (containing ~105mg SFN) inhibited HDAC activity significantly in peripheral blood mononuclear cells (PBMC) 3 and 6 hrs following consumption (104). Incubation of BPH-1, LnCaP, and PC3 prostate cancer cells with 15 μM SFN can significantly reduce HDAC expression (by 30%–40%), which is accompanied by a 50%–100% increase in acetylation of histones, as well as G2/M arrest of cell development and induction of apoptosis in a caspase-dependent manner (105). Upregulation of p21 gene expression by PEITC was found to be associated with chromatin remodeling, which compromises dynamic changes in both histone acetylation and methylation (106). To note, PEITC also exhibits the dual functions of CpG demethylation and HDAC inhibition and in epigenetic regulation of various genes (107, 108).

Cancer stem cells (CSCs) are characterized by their abilities in both self-renewal and differentiation, in which the phenotype is closely associated with cellular epigenetic regulations (109). It has been widely recognized that CSCs are implicated in initiation, drug resistance, and relapse of many types of cancer. Experimental results suggested that ITCs may target CSCs by increasing their sensitivity to chemotherapeutic agents, enhancing their differentiation, and inhibiting their self-renewal signaling (110, 111). In an *in vivo* xenograft model, daily continuously injection of 50mg/kg SFN for 2 weeks reduced

the $ALDH^+$ (a stem cell marker) subpopulation in SUM159 cells (112). In addition to the pro-apoptotic effect, SFN has also shown to effectively enhance human promyelocytic leukemia cells differentiation toward granulocytic and macrophagic lineage (113). PEITC also inhibits human CSCs proliferation *in vitro* (114). In addition, PEITC-mediated epigenomic alteration in LNCaP cells may give rise to floating sphere cells with increased H3K4 acetylation, DNMT1 down-regulation and GSTP1 activation. These spheres can differentiate to neuroendocrine cells (NEC) with decreased proliferation, expression of androgen receptor, and PSA under androgen deprivation (115).

1.6 Summary

Collectively, carcinogenesis is a multi-stage process that requires multi-targeted approaches for the development of an effective treatment regimen. Dietary phytochemicals are of particular interest in cancer prevention/therapy because of their generally low toxicity whereas they are able of targeting multiple pathways and mechanisms. Currently, there is an increasing interest on their epigenetic modulatory activities in gene regulation due to the reversibility of these mechanisms of action and transgenerational nature. It has been reported that many bioactive phytochemicals with a wide structure diversity are capable of concomitantly upregulating tumor suppressor genes, downregulating tumor promoters/oncogenes in cancer, although the underlying mechanisms remain to be further elucidated. On the other hand, prolonged oxidative stress and inflammation response could initiate chromatin remodeling and aberrant DNA methylation in cancer cells. Given that chemopreventive phytochemicals commonly possess anti-oxidant and anti-inflammatory properties, the goal of this graduate research is to explore the epigenetic events associated with oxidative and inflammatory pathways in cancer initiation and progression.

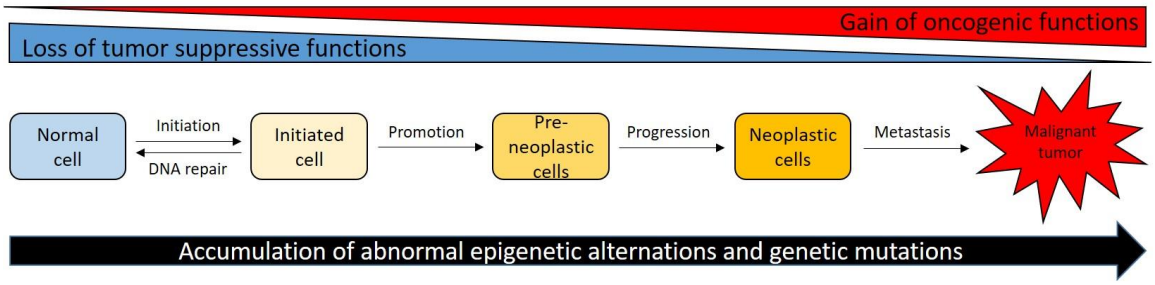

Figure 1.1 Cancer development is a multi-step process including initiation, promotion and progression.

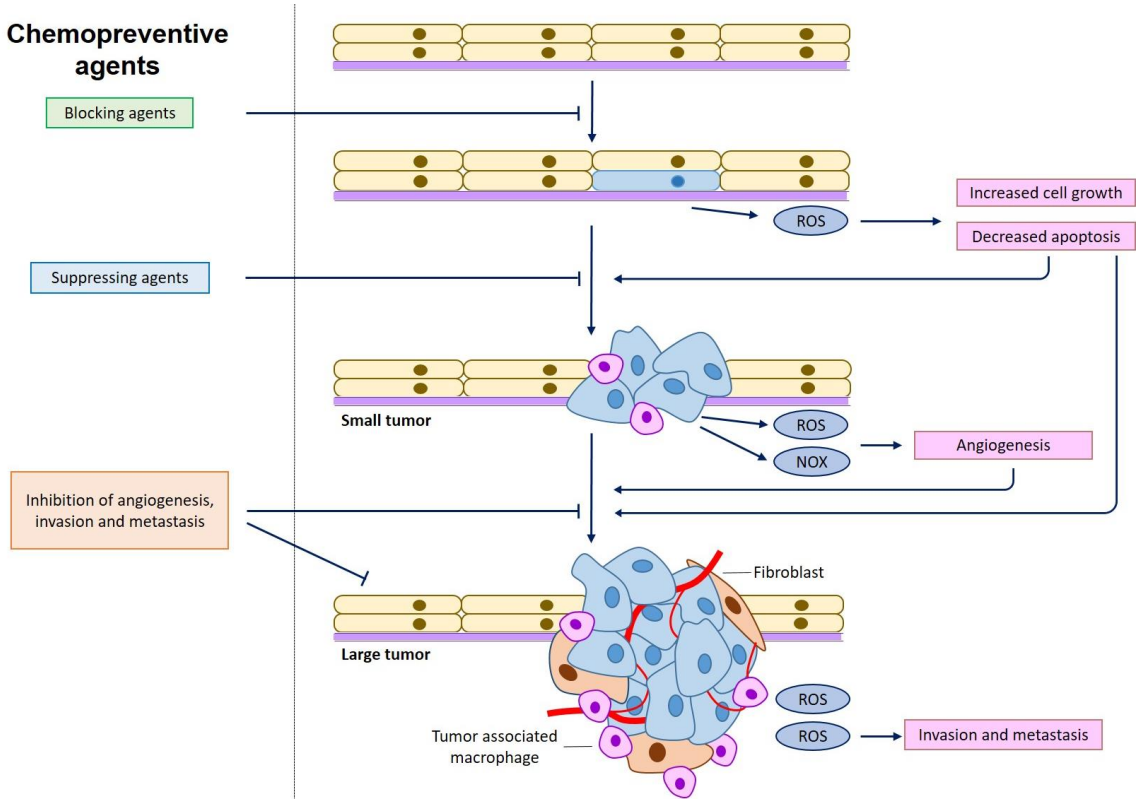

Figure 1.2 Intervention of tumor development processes by chemopreventive agents. Tumor initiation is triggered by carcinogen stimulation or irradiation in normal cells. Many phytochemicals can block this step by inducing the detoxification and antioxidant enzyme systems that protect cells from the damage caused by initiators. Chemopreventive agents can also block or retard the progress of tumor promotion and progression by modulating the key signaling pathways elicited by tumor promoters, inflammatory cytokines, growth factors, etc. A single chemopreventive agent could act as both tumor blocking and suppressing agent.

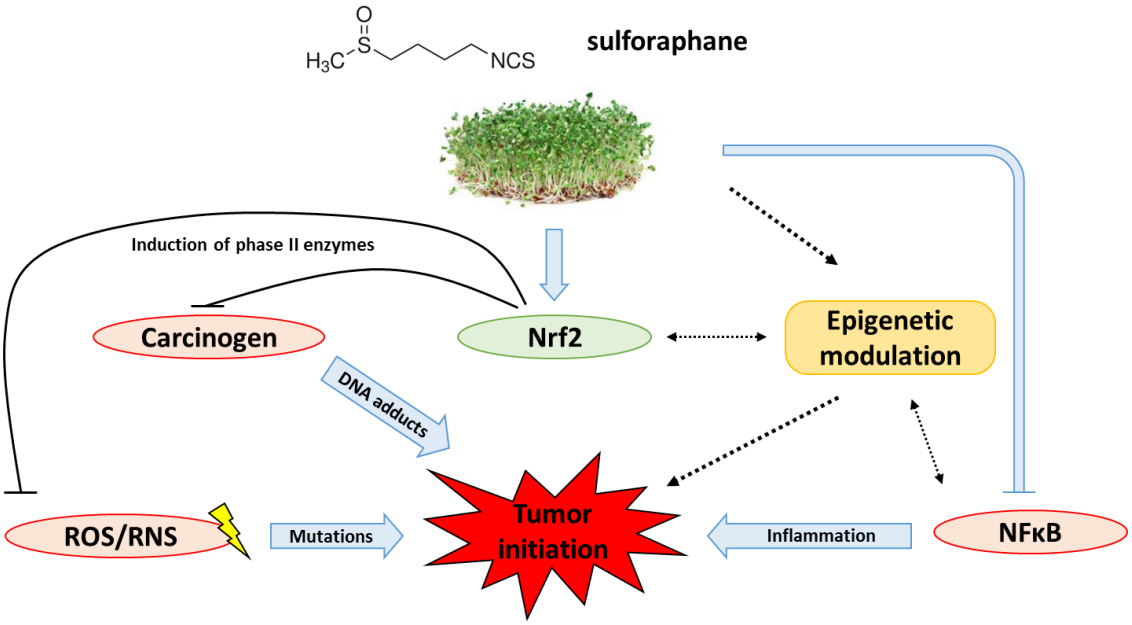

Figure 1.3 Integrating epigenetic mechanisms in cancer prevention using isothiocyanate sulforaphane. While anti-oxidant and anti-inflammatory effects of ITCs have been long considered as primary targets for chemoprevention, epigenetic mechanisms are emerging.

Chapter 2 Sulforaphane enhances Nrf2 expression in TRAMP C1 cells through epigenetic regulation[4,5,6]

2.1 Introduction

Prostate cancer is one of the most deadly cancers among men in the United States (116). It has been suggested that the incidence of prostate cancer is associated with increased intracellular oxidative stress (117-119), which is characterized by excess generation of reactive oxygen species (ROS) and/or reduction of antioxidant capacities. Chronic oxidative stress and associated pathological conditions such as inflammation had been demonstrated to drive genomic instability, genetic mutation and neoplastic transformation (120-123), thus it could serve as an essential factor in the development and progression of prostate cancer. Nuclear factor erythroid-2 related factor-2 (Nrf2 or NFE2L2), a helix-loop-helix basic leucine zipper transcription factor, is a key regulator in the cellular defense system against oxidative stress (124-128). Through its binding to antioxidant response element (ARE) in the promoter region (48, 129), activation of Nrf2 results in the induction of a series of anti-oxidative stress/detoxifying enzymes and proteins, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO-1), UDP-glucuronosyltransferases (UGT), and glutathione-S-transferases (GST) (130, 131) among others. It has been reported that Nrf2-deficient mice has an increased susceptibility towards carcinogen induced tumorigenesis (89, 90). Interestingly, our previous work found that the

[4] Part of this chapter has been published in Biochem Pharmacol. 2013 May 1;85(9):1398-404.
[5] **Key Words**: epigenetics, DNA methylation, sulforaphane, Nrf2, prostate cancer
[6] **Abbreviations**: ROS, reactive oxygen species; 5-aza, 5-azadeoxycytidine; TRAMP, transgenic adenocarcinoma of mouse prostate; MeDIP, Methylation DNA immunoprecipitation; ChIP, Chromatin immunoprecipitation; TSS, transcription starting site.

expression of Nrf2 and its downstream genes such as NQO1, UGT1A1, and GSTM1 are gradually down-regulated during the development of prostate tumor in TRAMP mice (132, 133). In addition to TRAMP mice, it was also reported that Nrf2 and members of GST mu family were distinctly decreased in human prostate cancer samples, which leads to extensive oxidative stress and DNA damage (134). Recently, we reported the attenuated expression of Nrf2 may be caused by epigenetic mechanism in prostate cancer of TRAMP mice and tumorigenic TRAMP-C1 cells (135).

Epigenetics is defined as changes in gene expression without alteration of DNA nucleotide sequences (136). Epigenetic regulation, particularly DNA methylation and histone modification, would change the interaction between gene promoters and transcription factors, resulting in either transcription promotion or repression. It has been reported that cancer progression is usually accompanied with epigenetic silencing of critical tumor suppressor genes through CpG island hypermethylation in their promoter region (137, 138). For instance in prostate cancer, coordinated hypermethylation of APC and GSTP1 can serve as a specific diagnostic marker in early stages of prostate cancer development (139, 140). In this context, a series of genes silenced through DNA methylation has been found in TRAMP prostate cancer and its derived cell lines (141, 142). Drugs which target on those enzymes responsible for epigenetic silencing, such as DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), could be useful in cancer prevention and therapeutic strategy. Treatment of 5-azadeoxycytidine (5-aza, a DNMT inhibitor) and trichostatin A (TSA, an HDAC inhibitor) has been shown to inhibit cell proliferation (143, 144). It has been reported 5-aza shows inhibitory effect towards prostate tumorigenesis in TRAMP mice *in vivo* (145). However, the side effects, such as toxicity

and nonspecific gene modulation limited their use as cancer chemopreventive/therapeutic agents (146).

Many compounds from our daily consumption of fruits and vegetables have been shown to exhibit cancer preventive effects through epigenetic mechanisms (147-149). It has been reported that epigallocatechin-3-gallate (EGCG) from green tea inhibits DNMTs (150) and histone acetyltransferases (HATs) (151). We have found that curcumin, a bioactive dietary component from turmeric, inhibits DNMTs activity potentially resulting in re-expression of Nrf2 in TRAMP C1 cell line (152). Sulforaphane (SFN), one of the most widely investigated isothiocyanates found in crucifers, has been shown to be a very potent cancer chemopreventive agent in numerous animal models of different cancers (81). First identified as a potent phase 2 detoxifying enzyme inducer, SFN has been shown to be a multi-targeted chemopreventive agent including inhibition of histone deacetylase (HDAC) activity (103), suggesting epigenetic alteration may be involved in the mechanism of chemoprevention by SFN. The aim of this study is to investigate the potential of SFN to reactivate the expression of Nrf2 through epigenetic regulation.

2.2 Materials and Methods

2.2.1 Cell culture and treatment

TRAMP C1 cells (generously provided by Dr. Barbara Foster, Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY) were maintained in DMEM with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Sulforaphane (SFN) was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Other chemicals, 5-azadeoxycytidine (5-aza) and trichostain A (TSA) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were seeded in 10 cm plates for 24 h and then treated with either 0.1% DMSO, 500 nM 5-aza or different concentrations of SFN in DMEM medium containing 1% FBS. The medium was changed every 2 days. On day 4, for the 5-aza and TSA combination treatment, 100 nM TSA was added to the 5-aza containing medium. Cells were harvested on day 5 for DNA, protein or total RNA extraction.

2.2.2 DNA extraction and bisulfite genomic sequencing

Genomic DNA was isolated from DMSO, SFN, or 5-aza/TSA treated TRAMP C1 cells using the QIAamp® DNA mini Kit (Qiagen, Valencia, CA). Then 500 ng genomic DNA was subjected to bisulfite conversion using EZ DNA Methylation Gold Kits (Zymo Research Corp., Orange, CA) following the manufacturer's instructions. The converted DNA was amplified by PCR using Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY) using primers that amplify the first 5 CpGs located between -1085 and -1226 of murine Nrf2 gene with the translation start site defined as +1. PCR products were cloned into pCR4 TOPO vector using a TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA). Plasmids from at least ten colonies of each treatment group were prepared using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and sequenced (Genwiz, Piscataway, NJ)

2.2.3 Methylation DNA immunoprecipitation (MeDIP) analysis

The MeDIP analysis was carried out using MagMeDIP Kit (Diagenode, Denville, NJ) according to the manufacturer's instruction with some modifications. Briefly, 5 μg DNA extracted from treated cells was adjusted to 50 μL in TE buffer and then sonicated in ice-cold water using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ) to shear the DNA to an average size of 300-500 base pairs (bp). The fragmented DNA was denaturated at 95°C

for 3 min and followed by immunoprecipitation with anti-methylcytosine antibody at 4℃ overnight. After incubation, the pulled-down DNA on magnetic beads were washed and digested with proteinase K and then isolated from beads. The primer set, sense 5'-TGA GAT ATT TTG CAC ATC CGA TA-3' and anti-sense 5'-ACT CTC AGG GTT CCT TTA CAC G-3', which covers the DNA sequence of the first 5 CpGs of murine Nrf2 was used for regular PCR and qPCR assays. For regular PCR, 1 μL of each MeDIP and input DNA was used as template for 35 cycles PCR amplification using Platinum PCR SuperMix kit (Invitrogen, Carlsbad, CA). The PCR products were then analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). For qPCR, the enrichment of MeDIP DNA was calculated according to the calibration of a series of dilution of input DNA, and the relative methylated DNA ratios were then calculated based on the control as 100% of methylated DNA.

2.2.4 RNA isolation and quantative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the treated cells using RNeasy Mini Kit (QIAGEN, Valencia, CA). Then first-strand cDNA was synthesized from 1 μg total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. To determine the RNA expression of specific genes, the cDNA was used as the template for real time PCR using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA), while β-actin was used as an internal loading control. The sequence of the primers used for real time PCR were as follows: Nrf2: sense 5'-AGC AGG ACT GGA GAA GTT-3' and anti-sense 5'-TTC TTT TTC CAG CGA GGA GA-3'; NQO1: sense 5'-AGC CCA GAT ATT GTG GCC G-3' and

anti-sense 5'-CCT TTC AGA ATG GCT GGC AC-3'; β-actin: sense 5'-CGT TCA ATA CCC CAG CCA TG-3' and anti-sense 5'-GAC CCC GTC ACC AGA GTC C-3'.

2.2.5 Protein lyses preparation and Western blotting

Treated cells from above were harvested using RIPA buffer supplemented with protein inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations of the cleared lysates were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Total proteins (20 μg) from each sample were separated by 4-15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Then the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) followed by blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. Then the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were visualized by SuperSignal enhanced chemiluminiscence (ECL) detection system and recorded using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). Primary antibodies were purchased from different resources: anti-Nrf2, anti-NQO1 and anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-DNMT1 and anti-DNMT3a from IMGENEX (San Diego, CA); anti-HDAC1, anti-HDAC2, anti-HDAC5, and anti-HDAC7 from Cell Signaling (Boston, MA).

2.2.6 Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assay was performed using MAGnify Chromatin Immunoprecipitation System (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Briefly, formaldehyde at a final concentration of 1% was added to fix the cells (~5.0×10^6 cells in 10-cm dish). After incubation at room temperature for

10 min, excess formaldehyde was quenched by 5 M glycine. After washing twice with PBS, cells were scraped, pelleted and then resuspended in Cell Lysis Buffer containing protease inhibitor cocktail. The samples were sonicated in ice cold water using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ) to shear the cross-linked DNA to an average length of 200-500 bp and centrifuged at 12,000 rpm to remove insoluble material. The chromatin solutions were then diluted using dilution buffer, and 10 μL of each was reserved as input control. Diluted chromatin solutions were then incubated overnight at 4℃ with protein A magnetic beads and specific antibody against anti-acetyl-Histone 3 (Ac-H3) (Millipore, Lake Placid, NY) or nonspecific Rabbit IgG. The immunoprecipitated complex-magnetic beads were collected using magnetic separator. After reverse cross-linking, DNA was purified according to manufacturer's instruction. Each purified DNA (1 μL) was used as template for regular PCR amplification using the same primers as those used for MeDIP analysis described above. The PCR products were then analyzed by agarose gel electrophoresis and visualized using EtBr staining. In parallel, qPCR assay was also performed to determine the amount of ChIP-purified DNA using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA).

2.2.7 Statistical analysis

Data are represented as the mean ± SD from three independent experiments at least. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Student's t-test to determine statistically significant difference among the means ($P < 0.05$).

2.3 Results

2.3.1 SFN decreases methylated CpG ratio in the promoter region of Nrf2 gene in TRAMP C1 cells

Our previous study showed that Nrf2 transscription was significantly suppressed when the first five CpGs in the promoter of Nrf2 gene were hypermethylated (135). Therefore, bisulfite sequencing was carried out to investigate if SFN treatment would demethylate the five CpGs of Nrf2. Figure 2.1 shows that the 5 CpGs were hyperrmethylated in the control group (methylation ratio 89.3%). Treatment with SFN (1.0 and 2.5 μM) and the combination of 5-aza (500 nM)/TSA (100 nM) after 5 days incubation reduced the methylation level to 56.0%, 38.7% and 46.7%, respectively. It suggested that SFN has demethylation potential on the promoter of Nrf2 gene.

3.3.2 SFN decreases the binding of anti-methyl cytosine antibody to the promoter region of Nrf2 gene in TRAMP C1 cells

MeDIP analysis was performed using anti-methyl cytosine antibody by immunoprecipitation and the enriched methylated DNA was then used as template for PCR amplification of the Nrf2 promoter region containing the first 5 CpGs. Both regular PCR (Figure 2.2A) and qPCR (Figure 2.2B) results show that SFN and the combination of 5-aza/TSA treatment significantly reduced the binding of the enriched Nrf2 promoter containing the first 5 CpGs to anti-methyl cytosine antibody (Figure 2.2).

3.3.3 SFN induces expression of Nrf2 and its downstream gene

Previously the expression of Nrf2 and its downstream genes has been shown to be dramatically decreased in TRAMP prostate cancer and tumorigeneric TRAMP C1 cell line

(132, 135). To examine if demethylation of the promoter region results in the transcription activation of Nrf2, the mRNA and protein expression levels of Nrf2 and its targeted gene NQO1 were measured. TRAMP C1 cells were treated with SFN for 5 days, and then qPCR was performed to evaluate the mRNA expression of Nrf2 and NQO1. The results indicated that both 1.0 and 2.5 μM SFN treatments increased Nrf2 and NQO1 mRNA expressions (Figure 2.3A). Consistently, protein expression of Nrf2 and NOQ1 was also significantly induced upon SFN treatment in TRAMP C1 cells (Figure 2.3B).

3.4 SFN alters expressions of epigenetics modifying enzymes

To further study the epigenetics mechanism underlying the demethylation effect of SFN on the Nrf2 promoter region, we next examined if SFN can alter the expression of those epigenetics modifying enzymes. DNA methylation is mediated by a family of DNA methyltransferases (DNMTs) which include a maintenance methyltransferase DNMT1 and two *de novo* methyltransferases DNMT3a and DNMT3b. As shown in Figure 2.4A, SFN decreased the protein levels of DNMT1 and DNMT3a in a dose-dependent manner in TRAMP C1 cells. However, no significant alteration was observed with the expression of DNMT3b.

In addition, SFN has been reported to be an HDAC inhibitor. Correspondingly, we found that SFN significantly decreased the protein levels of HDAC1, HDAC4, HDAC5, and HDAC7 in a dose-dependent manner (Figure 2.4B) while no considerable changes of HDAC2, HDAC3, and HDAC8 protein expressions. The global level of acetylated histone 3 (Ac-H3) was also highly induced by SFN treatment (Figure 2.4C). Next, chromatin immunoprecipitation (ChIP) assays were performed to further examine the association between Nrf2 gene promoter and Ac-H3 level after SFN treatments in TRAMP C1 cells.

In agreement with the observation of Ac-H3 level, the amount of immunoprecipitated DNA of Nrf2 promoter containing the first 5 CpGs fragment in SFN treatments (1.0 and 2.5 μM) was higher as compared to control treatments as shown in Figure 2.5.

2.4 Discussion

The imbalance between ROS and cellular antioxidant capacity could result in oxidative stress in organisms. Oxidative stress causes biochemical alterations of proteins, nucleic acids, and lipids in cells, which may further lead to inflammation or carcinogenesis (153). Nrf2, a transcription factor, plays an important role in regulating phase II antioxidant and detoxification enzymes which eliminate carcinogens and reactive intermediates and hence protect cells from oxidative stress induced damages (154-157). In recent studies of prostate cancer, low expressions of Nrf2 and its downstream target genes such as GST family are found in human (134) and in TRAMP prostate tumors (132). Accumulating evidence also shows that dietary phytochemicals can enhance the expressions of phase II antioxidant and detoxification enzymes mediated by the Nrf2 pathway (158). For example, γ-rich tocopherols can inhibit prostate carcinogenesis in TRAMP mice while it increases the expressions of Nrf2 and its target genes including UGT, GST, HO-1, and glutathione peroxidases (GPx) (132, 133). In addition γ-rich tocopherols could cause CpG demethylation in the Nrf2 promoter and higher Nrf2 expression in the prostate of TRAMP mice and in TRAMP C1 cells, potentially contributing to the prevention of prostate tumorigenesis (159). In addition, curcumin has been demonstrated to restore the epigenetically silenced Nrf2 gene through DNA demethylation in TRAMP C1 cells (152). Likewise, it has also been shown to possess cancer chemopreventive activity potentially through enhancing the expressions of Nrf2 and its downstream target genes (160, 161).

Furthermore, it has been demonstrated that broccoli sprouts containing abundant SFN can inhibit prostate tumorigenesis in TRAMP mice, which may be through the activation of Nrf2 pathway (162). Activation of Nrf2 pathway by SFN plays a role in chemoprevention in several animal models such as benzo[a]pyrene-induced forestomach tumors using wild-type and Nrf2-deficient ICR mice (163) and DMBA/TPA-induced skin carcinogenesis with wild-type and Nrf2-deficient C57BL/6 Mice (164). In our current study, SFN restores the expression of Nrf2 and its target gene NQO1 (Figure 2.3), via demethylating the CpGs of the promoter of Nrf2 gene in TRAMP C1 cells, may play a role in chemoprevention in TRAMP mouse model.

Epigenetic regulation plays a critical process in cancer development, which includes DNA methylation and histone modification. Cellular DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) which cause epigenetic silencing are the major targets of cancer prevention and therapeutic strategy. However, some anti-cancer drugs including 5-azacitdine and 5-aza-2'-deoxycytidine (DNMTs inhibitors) and vorinostat, belinostat, romidepsin, and panobinostat (HDAC inhibitors) may have adverse side effects (165-167). Many studies have demonstrated that some dietary phytochemicals, such as epigallocatechin-3-gallate (EGCG) (151), curcumin (152), and genistein (168), with cancer chemopreventive and/or anti-cancer activities have epigenetic regulation potentials. The epigenetic effect of SFN on gene transcription has also been recently reported. SFN may cause site-specific CpG demethylation of the first exon of hTERT gene through the suppression of DNMT1 and DNMT3a in a dose- and time-dependent manner in human breast cancer cells (169). In our present study, we also found that SFN treatment exhibited hypomethylation effect on the first 5 CpGs in the Nrf2 promoter in TRAMP C1 cells

(Figure 2.1). This hypomethylation effect is supported by MeDIP assay showing that SFN significantly decreased the amount of the Nrf2 promoter containing the first 5 CpGs using anti-mecyt antibody (Figure 2.2). These results might be associated with the suppression of the protein expression of DNMT1 and DNMT3a (Figure 2.4A). With respect to HDACs, it has been reported that SFN as well as its two major metabolites, SFN-cysteine and SFN-*N*-acetylcysteine, can suppress HDAC activity *in vitro* (170). SFN also shows a similar HDAC inhibitory effect in BPH-1, LnCaP and PC-3 prostate epithelial cells (105). It has been reported that the levels of the active chromatin markers (acetyl-H3, acetyl-H3K9 and acetyl-H4) are increased, but the levels of the inactive chromatin markers (trimethyl-H3K9 and trimethyl-H3K27) are decreased in MCF-7 and MDA-MB-231 cells after SFN treatment (169). In *in vivo* study, the suppressive effect of SFN against the growth of PC-3 xenografts might be mediated by inhibiting HDAC activity (104). SFN can also retard intestinal carcinogenesis in Apc-minus mice through histone modification accompanied with inhibition of HDAC activity in the colonic mucosa and polyps (171). In addition, HDAC activity in peripheral blood mononuclear cells (PBMC) was inhibited in the human subjects after 3 and 6 hrs following consumption of SFN-rich broccoli sprouts (104). Our present results show that the protein expression levels of HDAC1, HDAC4, HDAC5, and HDAC7 were significantly decreased in a dose-dependent manner while the Ac-H3 protein level was highly induced by SFN (Figure 2.4B & C). Furthermore, we also demonstrated that SFN treatment increased the binding of Ac-H3, one of the transcriptionally active chromatin markers, to the promoter of Nrf2 gene (Figure 2.5). It has also been showed that SFN can interfere with the formation of a transcriptional regulator complex which consists of methyl-CpG binding proteins, DNMT and HDAC (172). The release of the co-repressor

complex could result in the concomitant enrichment of acetyl histones including Ac-H3. Therefore, our current findings are in agreement with previous investigations and support the evidence of epigenetic effect of SFN in restoring Nrf2 expression, including DNA demethylation and chromatin remodeling process.

2.5 Conclusion

SFN has been shown to inhibit prostate carcinogenesis in TRAMP mice (173). Gradual down-regulation of Nrf2 and Nrf2 downstream target genes during the progression of prostate tumorigenesis in TRAMP mice has been reported (132), and SFN-rich broccoli sprouts increases the expression levels of Nrf2 and its downstream target genes and inhibits tumorigenesis (162). In our current study, SFN is an epigenetic regulator and restores the silenced Nrf2 gene in TRAMP C1 cells through DNA demethylation and histone modifications. These finding suggest that SFN epigenetically restore the expression of Nrf2 and Nrf2 downstream antioxidant and detoxification related genes during the initiation, progression and development of prostate cancer in TRAMP mice may play a role in SFN's chemoprevention effects.

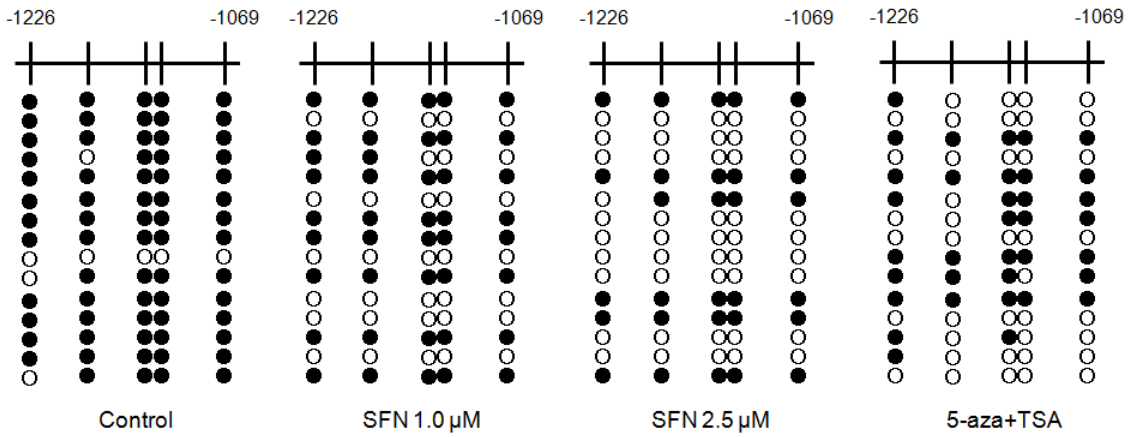

Figure 2.1 Effect of SFN on methylation status of the Nrf2 promoter regions in TRAMP C1 cells. Cells were treated with 1.0 or 2.5 μM SFN for 5 days, and then genomic DNA was extracted from the treated cells. A combination treatment of 5-aza (500 nM)/TSA (100 nM) was used as positive control, while TSA was added 24 h before cell collection. The methylation pattern of the first five CpGs located at -1226 to -1085 in Nrf2 promoter region, where the translational starting site is considered as +1 was determined. Bisulfite genomic sequencing was performed as described in Materials and Methods. Black dots indicated methylated CpGs while open circles indicate unmethylated CpGs. At least ten clones were picked randomly and sequenced from each of the three independent experiments, and fifteen clones were selected based on the relative frequency of methylation from each treatment.

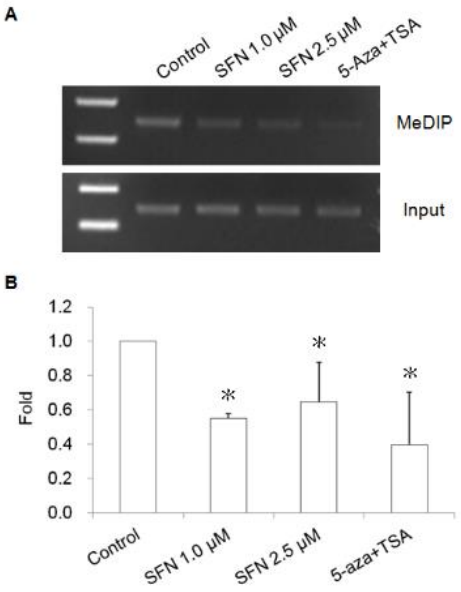

Figure 2.2 Effect of SFN on methylation of Nrf2 promoter region in TRAMP C1 cells using methylation DNA immunopredipitation (MeDIP) assay. Genomic DNA (10 μg) extracted from SFN or 5-aza/TSA treated TRAMP C1 cells were used for MeDIP analysis. Genomic DNA were sonicated, denatured and subjected to DNA immuniprecipitation (IP) with anti-methyl cytosine antibody. Then regular PCR (A) and qPCR (B) were performed to analyze the enrichment of methylated fragments using the primers covering the DNA sequence containing the first five CpGs in promoter region of the Nrf2 gene. (A) Regular PCR was performed to compare the immunoprecipitated DNA with their inputs, a representative result is shown from three independent experiments. (B) The enrichment of the MeDIP DNA was determined by qPCR on the basis of the standard curve from a serial dilution of the inputs. Relative methylated ratio was calculated by comparing with control group as 100% methylated DNA. Data are expressed as mean ± SD from four independent experiments. * Different from control, $P < 0.05$.

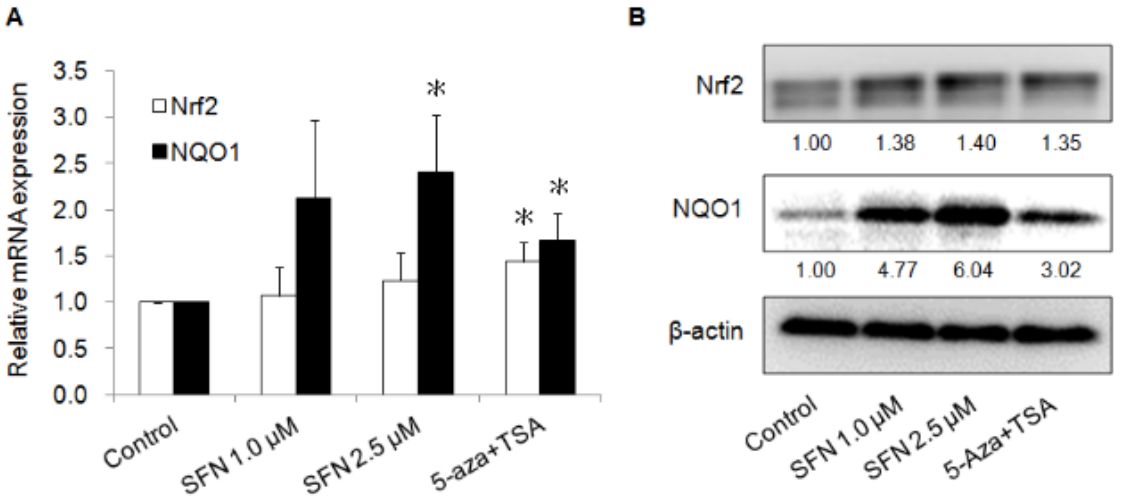

Figure 2.3 Effect of SFN on the mRNA (A) and protein (B) expressions of Nrf2 and NQO-1. After 5 days of treatment, mRNA and protein were isolated from TRAMP C1 cells as described above. The mRNA and protein levels of Nrf2 and NQO-1 were determined using qPCR and Western blotting, respectively. (A) The mRNA level was determined using ABI7900HT qPCR system. Data are expressed as mean ± SD from three independent experiments. * Different from control, $P < 0.05$. (B) For protein analysis, the bands which were bound with specific Nrf2 and NQO1 antibodies were visualized using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA) and quantified using Quantity One software. β-Actin was used as an equal loading control.

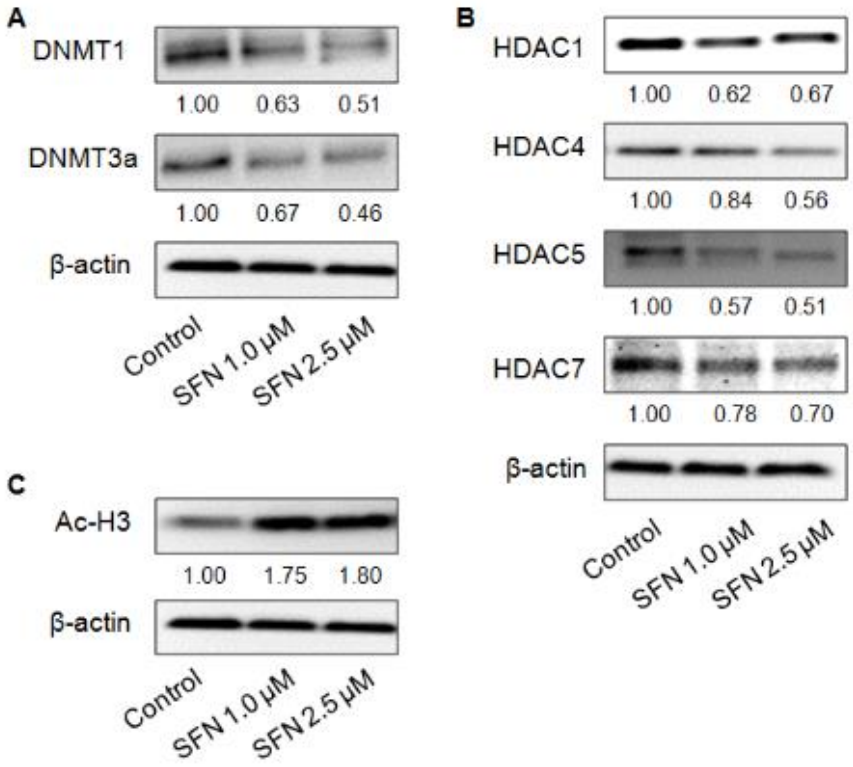

Figure 2.4 Effect of SFN on the protein expressions of DNMTs (A), HDACs (B), and acetylated histone 3 (Ac-H3) (C) in TRAMP C1 cells. Cell lysates were prepared after SFN with the indicated doses for 5 days, followed by Western blotting to analyze protein expressions of DNMTs, HDACs, and Ac-H3 using specific antibodies. β-Actin was used as an equal loading control.

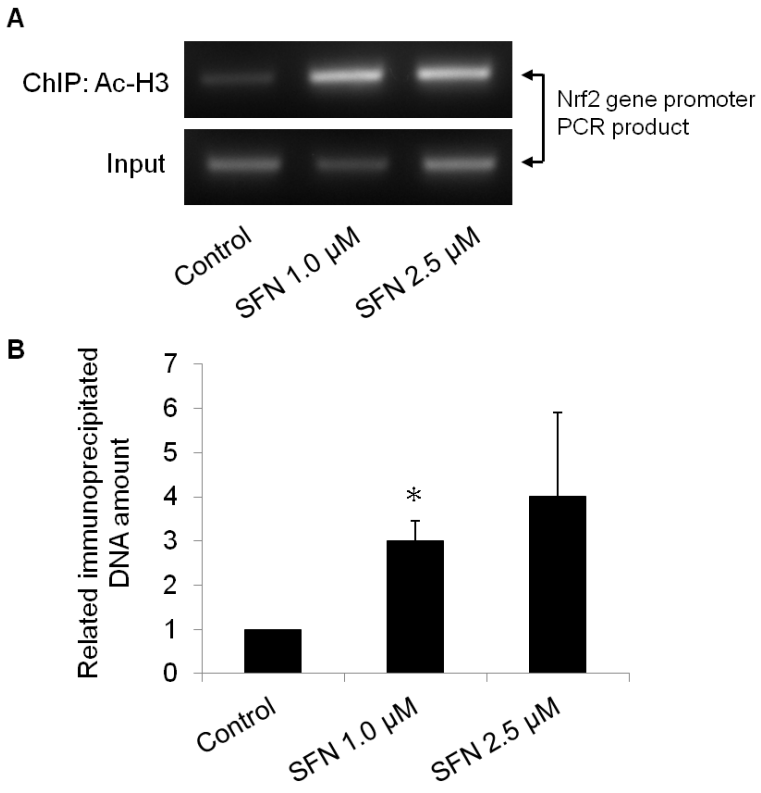

Figure 2.5 Effect of SFN on the binding of Nrf2 promoter region to acetylated histone 3 (Ac-H3). ChIP assay was performed as described in Material & Methods using antibody against Ac-H3. Regular PCR (A) and qPCR (B) were performed to analyze the enrichment of DNA binding to Ac-H3 using primers which amplify the DNA sequence containing the first five CpGs in the promoter region of Nrf2 gene. (A) Regular PCR was performed to compare the immunoprecipitated DNA versus their inputs of each treatment, and a representative result is shown from three independent experiments. (B) The enrichment of the Ac-H3 binding DNA was determined by qPCR based on the standard curve which was obtained from a serial dilution of the inputs. Relative ratio was calculated by the fold change compared to control group. Data are expressed as mean ± SD from three independent experiments. * Different from control, $P < 0.05$.

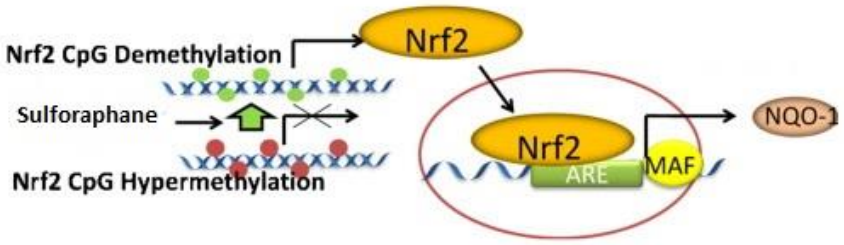

Figure 2.6 Summary of Chapter 2

Chapter 3 Blocking of JB6 cell transformation by Tanshinone IIA: Epigenetic re-activation of Nrf2 anti-oxidative stress pathway[7,8,9]

3.1 Introduction

The incidence of skin cancer has risen steadily in recent years, and an estimated 82,770 new cases will occur in the United States during 2013(174). It is evident that oxidative stress plays an important role in carcinogenesis and cancer progression(175). Oxidative stress is caused by an imbalance between systemic reactive oxygen species (ROS) and the body's capability to neutralize ROS, which may result in genomic instability, genetic mutation and neoplastic transformation, leading to a higher incidence of carcinogenesis. Endogenous ROS can be generated during normal cellular metabolism, immune reactions or under pathological conditions, whereas exogenous ROS may originate from the exposure to air pollution, UV irradiation, microorganisms, viruses and xenobiotics. Skin is the first defensive barrier for the body and is thus susceptible to both endogenous and exogenous ROS(176).

It is estimated that more than 2 million skin cancers around the world could be prevented by protecting skin from excessive ROS exposure, such as extensive sun exposure and indoor tanning. To protect the body from ROS-mediated cellular injury, elimination or neutralization of ROS is required to maintain the redox balance in the cell(177). Nuclear

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[7] Part of this chapter has been published in AAPS J. 2014 Nov;16(6):1214-25.
[8] **Key Words**: epigenetics, Nrf2, skin cancer, tanshinone IIA
[9] **Abbreviations**: TIIA, tanshinone IIA; TCM, traditional Chinese medicine; shRNA, short hairpin RNA; TPA, 12-O-tetradecanoylphorbol-13-acetate; TSA, Trichostatin A.

factor erythroid-2-related factor-2 (Nrf2 or NFE2L2) is a critical molecule involved in maintaining a balanced redox potential in the human body and plays an important role in regulating the expression of phase II detoxifying and anti-oxidative enzymes (29). Nrf2 or NFE2L2 is a basic helix-loop-helix leucine zipper transcription factor that can activate antioxidant genes by binding the antioxidant response element (ARE) in their corresponding promoter areas(178). These enzymes that protect against oxidation include heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase-1 (NQO-1), UDP-glucuronosyltransferase (UGT) and glutathione-*S*-transferase (GST) among others. It has been reported that Nrf2-deficient mice display increased susceptibility to tumorigenesis induced by a carcinogen, and cancer chemoprevention is partially correlated with the induction of phase II enzymes. The Nrf2 -/- mice were found to have a higher risk of developing skin cancer when treated with DMBA-TPA (164).

Our recent study demonstrated that the expression of Nrf2 can also be regulated by epigenetic alterations in both the prostate tissue of the transgenic adenocarcinoma mouse (TRAMP) and tumorigenic TRAMP C1 cells (135, 179). Epigenetic modifications, defined as the regulation of gene expression without an alteration in the DNA sequence, include DNA methylation, histone modification, nucleosome remodeling and microRNA silencing and have been reported to contribute to many diseases(180). Accumulating evidence suggests that carcinogenesis can be modulated by epigenetic alterations such as DNA methylation and histone modifications in tumor suppressor genes and oncogenes(181). DNA methylation typically occurs at CpG sites(182, 183). Physiologically, 70% to 80% of CpG sites within promoters are methylated, resulting in gene silencing. In contrast, the hypomethylation of CpG sites has been associated with the

overexpression of target genes. To maintain normal methylation status, some co-suppressors, including DNA (cytosine-5)-methyltransferase (DNMT), histone deacetylases (HDACs), methyl CpG binding protein 2 (MECP2), the transcriptional repressor sin3 and hBrm, are also involved (184). Among the repressors, DNMTs and HDACs are considered as the two major epigenetic effectors for the transcriptional control of gene promoters. Drugs targeting the enzymes responsible for epigenetic silencing, such as 5-azadeoxycytidine (5-aza, a DNMT inhibitor)(185) and trichostatin A (TSA, an HDAC inhibitor), have been widely investigated as cancer therapeutic agents. In addition to western drugs, phytochemicals have also been found to possess chemoprevention effects via epigenetic alterations(186). For example, mahanine, a carbazole alkaloid found abundantly in some Asian vegetables, has been reported to be a DNA hypomethylation agent that restored the expression of an epigenetically silenced tumor suppressor gene due to the inhibitory effect of DNMTs(187).

Danshen, the rhizome of *Salvia miltiorrhiza* Bunge, is one of the most widely used Chinese herbs. Danshen has been used for centuries primarily in traditional Chinese medicine (TCM) without obvious side effects for the treatment of coronary heart disease (188). The pharmacological activities of Danshen include anticancer, antioxidant, anti-inflammatory and antibacterial activity among others. Two major groups of compounds, phenolic acids such as salvianolic acid and lithospermic acid B, and tanshinones such as tanshinone I, tanshinone IIA and cryptotanshinone, have been identified in *Salvia miltiorrhiza*. Among these components, tanshinone IIA (TIIA) is the major constituent that is officially used as a quality control marker as per Chinese Pharmacopoeia (Figure 1) and was found to be the major antioxidant component in *Salvia miltiorrhiza*. A recent study

showed that TIIA was able to modulate the intracellular redox status in human aortic smooth muscle cells by inducing Nrf2(189).

As mentioned above, overexposure to ROS may cause cellular injury, leading to a higher incidence of cancer. TIIA may be able to protect the cell from ROS-induced carcinogenesis by activating the Nrf2 pathway through epigenetic modulation. Thus, here, an in vitro study was performed to investigate the potential inhibitory effect of TIIA on the neoplastic transformation of JB6 P+ cells (a mouse keratinocyte cell line) when exposed to a carcinogen and to determine the underlying epigenetic mechanisms.

3.2 Materials and Methods

3.2.1 Materials

Tanshinone IIA (TIIA), 5-azadeoxycytidine (5-aza), bacteriological agar, Eagle's basal medium (BME), puromycin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and trichostatin A (TSA) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), minimum essential medium (MEM) and trypsin-EDTA solution were purchased from Gibco Laboratories (Grand Island, NY). The primary antibodies anti-Nrf2, anti-HO-1, anti-NQO-1, anti-UGT1A1 and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies anti-DNMT (DNMT1, DNMT3a and DNMT3b) were obtained from IMGENEX (San Diego, CA), and the primary antibodies anti-HDAC (HDAC1, HDAC2, HDAC3, HDAC4 and HDAC8) were obtained from Cell Signaling (Danvers, MA).

3.2.2 Anchorage-independent cell neoplastic transformation assay

Stable Mock (vector control) or Nrf2-knockdown JB6 P+ cells were established using lenti-virus mediated short hairpin RNAs (shRNAs) as previously described(102). Both control and Nrf2-depleted JB6 P+ cells were used in the following TPA-induced neoplastic transformation assay. The JB6 P+ cells were transferred to 1 mL of BME containing 0.33% agar over 3 mL of BME containing 0.5% agar with 10% FBS in 6-well plates. The cells were maintained with TPA (20 ng/mL) alone or in a combination with TIIA. The cell colonies that formed in soft agar were photographed using a computerized microscope system with the Nikon ACT-1 program (Version 2.20, LEAD Technologies, Charlotte, NC) and counted using the ImageJ program (Version 1.40g, National Institutes of Health).

3.2.3 Cell culture and treatment

The human hepatocellular HepG2-C8 cell line was previously established by stable transfection with an ARE-luciferase construct. The cells were cultured and maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. JB6 P+ cells from American Type Culture Collection (Manassas, VA) were maintained in MEM containing 5% FBS with the same concentration of antibiotics. JB6 P+ cells stably transfected with shNrf2 were maintained in MEM supplemented with 5% FBS and 2 μg/mL puromycin. The cells were incubated at 37ºC in a humidified 5% CO_2 atmosphere. DMSO was used as a vehicle in all experiments at a concentration of 0.1%.

3.2.4 Cell viability tests

JB6 P+ cells, JB6-shNrf2 and HepG2 were seeded in 96-well plates at density of 6 × 10^3 cells/well. After incubation for 24 h, the cells were treated with either vehicle or various concentrations of TIIA for another 5 days. The medium was changed every 2 days. Cell viability was determined using a CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

3.2.5 Luciferase reporter activity assay

The stably transfected HepG2-C8 cells expressing the ARE-luciferase vector were used to study the effects of TIIA on Nrf2-ARE pathways. The ARE-luciferase activity in the HepG2-C8 cells was determined using a luciferase assay kit in accordance with the manufacturer's instructions (Promega, Madison, WI). Briefly, HepG2-ARE-C8 cells (1.0 × 10^5 cells/well) were seeded in 12-well plates in 1 mL of medium containing 10% FBS, incubated for 24 h and then treated with various concentrations of samples. Afterwards, the cells were lysed using the reporter lysis buffer, and 10 μL of the cell lysate supernatant was analyzed for luciferase activity using a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Normalization of the luciferase activity was performed based on protein concentrations, which were determined using a BCA protein assay (Pierce Biotech, Rockford, IL, US). The data were obtained from three independent experiments and are expressed as the inducible fold change compared with the vehicle control.

3.2.6 RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

JB6 P+ cells were seeded in 6-cm dishes at a density of 1 × 10^4 cells/dish. After incubation for 24 h, the cells were treated with TIIA at different concentrations for 5 days.

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The SuperScript III First-Strand cDNA Synthesis System (Invitrogen, Grand Island, NY) was used to synthesize first-strand cDNA. The mRNA expression of Nrf2, HO-1, NQO1, HDACs (HDAC1, 3, 4, and8) and DNMTs (DNMT1, 3a, and 3b) was determined using quantitative real-time PCR (qPCR). The primer pairs used were described previously.

3.2.7 Western blotting

After incubation for 24 h, JB6 P+ cells (5×10^3 cells per 6 cm dish) were treated with various concentrations of TIIA. Protein was extracted using RIPA buffer (Cell Signaling Technology®, Danvers, MA), and the protein concentration was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). The proteins were separated by 4 to 15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 buffer, the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The SuperSignal enhanced chemiluminescence (ECL) detection system was used to detect the antibody-bound proteins on the membrane. The intensity of the bands was analyzed using densitometry and the ImageJ program (Version 1.40g, National Institutes of Health).

3.2.8 HDAC and DNMT activity assay

HDAC and DNMT activity assays were performed using the EpiQuik™ HDAC Activity/Inhibition Assay Kit and DNMT Activity/Inhibition Assay Kit (Epigentek, Farmingdale, NY) following the manufacture's protocol, respectively. The nuclear protein fraction was prepared using the NEPER Nuclear and Cytoplasmic Protein Extraction Kit

(Thermo scientific, Pittsburgh, PA), and the relative HDAC or DNMT activity was calculated based on the ratio of the HDAC or DNMT activity of the TIIA treatment group compared with that of the control group.

3.2.9 DNA isolation and bisulfite genomic sequencing

After incubation for 24 h, the cells were treated with TIIA at various concentrations or 5-aza (500 nM) in MEM containing 1% FBS for 5 days, and the medium was refreshed every 48 hours. For combination 5-aza and TSA treatment, TSA (100 nM) was added to the medium on day 4. The cells were harvested on day 5. Genomic DNA was isolated from the treated cells using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA). Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Gold Kit (Zymo Research Corp., Orange, CA). The DNA fragment containing the first 5 CpGs, which are located between -1085 and -1226 in the murine Nrf2 gene (with the translation start site defined as position +1), was amplified from the converted DNA by PCR using Platinum Taq DNA polymerase (Invitrogen, Grand Island, NY). The primer sequences were as follows: sense, 5'-AGT TAT GAA GTA GTA GTA AAA A-3'; anti-sense, 5'-AAT ATA ATC TCA TAA AAC CCC AC-3'. The PCR products were further cloned into a pCR4 TOPO vector using the TOPO™ TA Cloning Kit (Invitrogen, Carlsbad, CA). At least 10 colonies from each treatment group were randomly selected, and the plasmids were extracted using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The target region was analyzed by sequencing (GeneWiz, South Plainfield, NJ).

3.2.10 Chromatin Immunoprecipitation (ChIP) Assay

After treatment with various concentrations of TIIA or TSA (100nM), 1×10^7 cells in 150mm dish were cross-linked with 1% formaldehyde for 10 min at room temperature.

Then 1.25M glycine was added to quench the excess formaldehyde. Next, the cells were washed twice with cold PBS and pelleted by spinning down at 1000×g for 5 min. Chromatin samples were prepared using EpiSeeker Chromatin Extraction kit (Abcam, Cambridge, MA), then sheared to an average length of 600-1000 bp using a Bioruptor sonicator (Diagenode, Sparta, NJ). Cross-linked chromatin fragments were subjected to immunoprecipitation with specific antibodies for Ac-H3 and Pol II (Cell Signaling, Danvers, MA) or non-specific IgG using EpiSeeker ChIP-one step kit (Abcam, Cambridge, MA) following manufacturer's protocol. After precipitation, 2µl of each purified DNA was used as template for 35 cycles of PCR amplification using primers that cover the first 5 CpG: sense: 5'-GAG GTC ACC ACA ACA CGA AC-3'; anti-sense, 5'-ATC TCA TAA GGC CCC ACC TC-3'. Another set of primers which cover the transcription start site was used to evaluate the recruitment of RNA polymerase complex II to the Nrf2 promoter: sense, 5'-CCT CAC CTC TGC TGC AAG TA-3'; anti-sense, 5'-GGC AAC TCC AAG TCC ATC AT-3'. The PCR products were analyzed by 2.0% agarose gel electrophoresis and visualized using EB staining. Primers covering GAPDH promoter region was used as a control to verify the efficacy of ChIP assays.

3.2.11 Statistical analysis

The data are presented as the means ± SDs. Statistical analyses were performed using one-way analysis of variance (ANOVA). $P < 0.05$ or $P < 0.01$ was considered to be statistically significant.

3.3 Results

3.3.1 TIIA inhibits TPA-induced JB6 P+ cell transformation

The effects of TIIA treatment on the TPA-induced anchorage-independent growth of JB6 P+ and JB6-shNrf2 cells were examined in soft agar. TIIA treatment with concentrations ranging from 0.5 to 5 μM significantly decreased the number of JB6 P+ colonies compared with the TPA-treated control group (positive control) (P<0.01, 1-way ANOVA) (Figure 3.2). Treatment with TIIA (1.0-5.0 μM) significantly inhibited the TPA-induced anchorage-independent growth of JB6 P+ cells by approximately 28%-56%, indicating that TIIA may have a chemopreventive potential against TPA-induced carcinogenesis in JB6 P+ cells.

The colony formation of JB6-shNrf2 cells in soft agar was significantly increased when compared with the vector control cell line (Figure 3.2) ($P < 0.01$, Student's t-test). In addition, no significant difference was observed between control and TIIA treatment groups ($P>0.05$, 1-way ANOVA). These results indicate that the protective effect of TIIA in JB6-shNrf2 cells was decreased, suggesting that Nrf2 plays an important role in the inhibitory effects of TIIA on TPA-induced JB6 P+ cell transformation.

3.3.2 Cytotoxicity of TIIA in JB6 P+ and HepG2-C8 cells

The viability of JB6 P+, JB6-shNrf2 and HepG2-C8 cells after TIIA treatment for 24 h is shown in Figure 3.3; IC_{50} values of 28.9 μM, 20.1 μM and 26.3 μM for TIIA in JB6 P+, JB6-shNrf2 and HepG2-C8 cells, respectively, were observed. Similar cytotoxicity levels were observed among these three cell lines. To avoid using concentrations that were

substantially toxic, no concentration greater than the IC_{50} was utilized in subsequent cell studies.

3.3.3 TIIA induces ARE-luciferase reporter activity

The relative fold changes of luciferase activity in cells transfected with the ARE-luciferase reporter vector are shown in Figure 3.4. TIIA induced luciferase activity in a dose-dependent manner at concentrations ranging from 5 to 25 μM, although no inductive effect was observed at concentrations lower than 5 μM. This result further verified the effect of TIIA on Nrf2 as reported previously.

3.3.4 TIIA upregulates the mRNA and protein levels of Nrf2 target enzymes in JB6 P+ cells

TIIA treatment significantly increased the expression of Nrf2, NQO1 and HO-1 mRNA (Figure 5A). The upregulation of HO-1 and Nrf2 occurred in a dose-dependent manner at concentrations ranging from 2 to 10 μM. Conversely, the upregulation of NQO1 mRNA occurred in a dose-independent manner, with the highest expression level found at the lowest concentration (2 μM). This inconsistency may be caused by experimental variability or the incubation time. Another possibility is that TIIA may inhibit the expression of NQO1 at higher doses. In contrast, no statistically significant effect of TIIA was observed on UGT1A1, which was slightly decreased by the treatment with TIIA compared with the control group.

The protein levels of Nrf2, HO-1, NQO1 and UGT1A1 were further evaluated in the JB6 P+ cells treated with TIIA using Western blotting. In accordance with the qRT-PCR results, TIIA (2-10 μM) also increased the protein levels of Nrf2, HO-1 and NQO1 in

a concentration-dependent manner (Figure 3.5B). Unlike the mRNA result, the expression of UGT1A1 at the protein level was also increased. These results indicate that TIIA has the potential to induce the Nrf2 pathway, including antioxidant and detoxifying enzymes, which might be correlated with the increased cellular expression of Nrf2 in JB6 P+ cells.

3.3.5 TIIA inhibits the mRNA and protein expression of epigenetic modification enzymes in JB6 P+ cells

The effect of TIIA on DNMTs (subtypes of DNMT1, 3a, and 3b) and HDACs (subtypes of HDAC1, 3, 4, and 8) was further examined to investigate the epigenetic mechanism by which TIIA affects promoter demethylation and induces Nrf2 gene transcription. TIIA at concentrations of 2.0-10.0 μM decreased the mRNA of HDAC1, 3 and 8 as well as DNMT1, 3a and 3b in a concentration-dependent manner in JB6 P+ cells after five days of treatment (Figure 3.6A). Consistent with the mRNA expression, TIIA also decreased protein levels (Figure 3.6B) in a concentration-dependent manner at concentrations ranging from 2-10 μM. In addition, TIIA repressed the expression of the HDAC4 protein, although no effect was observed at the mRNA level. These results indicate that TIIA has the potential to epigenetically modify DNA methylation of the Nrf2 promoter; this may be an important mechanism for the induction of Nrf2 as mentioned above.

3.3.6 HDAC and DNMT activity assay

Treatment with either 5.0 μM or 10.0 μM TIIA significantly inhibited relative HDAC activity by 50% ($P < 0.01$) (Figure 3.7). However, no significant inhibition of DNMT activity was observed despite the inhibition of DNMT1, 3a, and 3b expression by TIIA at the concentration mentioned above.

3.3.7 TIIA decreases the proportion of methylated CpG in the Nrf2 gene promoter region

The Nrf2 promoter region containing the first five CpGs was converted and amplified. The methylation status of the CpGs was then determined using bisulfite sequencing. Hypermethylation of these five CpGs (84%) was observed in the control JB6 P+ cells (Figure 3.8), which was consistent with previous reports. In contrast to the control group, the methylation level decreased to 46.0% when cells were treated with TIIA (10 μM), which was similar to the positive control group (46.0%, 5-aza (500 nM) + TSA (100 nM)). These results suggest that TIIA reverses the CpG methylation status of the Nrf2 gene promoter, which may drive the transcriptional re-expression of Nrf2 in JB6 P+ cells.

3.3.8 TIIA increases the recruitment of RNA polymerase complex II at Nrf2 transcription start site.

ChIP assays were employed to further examine the proteins that could be potentially interacted with Nrf2 promoter in JB6 cells. Based on the methylation pattern of Nrf2 promoter, primers are designed to cover the first 5 CpGs and the TSS to detect the enrichment of Ac-H3 and RNA polymerase II associated with the Nrf2 promoter. Although TIIA treatment slightly increased total Ac-H3 level (Fig. 9B), an enriched acetylation of H3 was not observed at the loci where the first 5 CpGs locate. On the other hand, the recruitment of Pol II to Nrf2 TSS was significantly increased after exposure of TIIA (Fig 3.9D). The specificity of ChIP assays were verified by nonspecific IgG pull down. As a positive control, GAPDH promoter is equally amplified among all the samples associated with Pol II.

3.4 Discussion

In this study, the chemopreventive potential of TIIA in skin cancer and its underlying mechanisms were investigated in an in vitro study using JB6 P+ cells. JB6 P+ is a normal skin keratinocyte cell line that will transform under carcinogenic or environmental challenges including TPA, EGF and UVB(190). Therefore, this model has been widely used for skin cancer risk assessment. In the present work, we found that TIIA inhibited TPA-induced JB6 P+ cell transformation, which indicated a potential chemopreventive effect of TIIA on skin cancer.

Nrf2 is an important transcription factor regulating phase II detoxifying (or cytoprotective) enzymes and the expression of antioxidant-response genes. Numerous studies have provided strong evidence for a relationship between Nrf2 deficiency and increased susceptibility to carcinogenesis, and elimination of carcinogens through induction of cytoprotective enzymes is one important approach to prevent cancer(190).In previous study, we have shown that the epigenetics re-activation of Nrf2 pathway appears to be an important mechanism resulting in the inhibition of transformation of JB6 P+ cells(102). Therefore, Nrf2 is a key molecular target for the development of chemopreventive agents. A large amount of chemicals, especially those from natural products such as dithiolethiones and sulforaphane, have been extensively studied for their effects on the induction of the Nrf2 pathway(183, 191). Several mechanisms have been identified, including an interaction between KEAP1 and NRF2, phosphorylation of Nrf2 by various protein kinases, interaction with other protein partners (p21, caveolin-1) and epigenetic regulation. Among these mechanisms, epigenetic changes such as DNA promoter methylation and chromatin modifications have been found to play an important

role in tumorigenesis(178). Nrf2 loss of function, which is associated with promoter hypermethylation, was reported in skin and prostate tumorigenesis(192). The methylation of the first five CpG sites in the Nrf2 promoter has been shown to substantially decrease Nrf2 expression. *Radix Angelicae* Sinensis extract and Z-ligustlide (one active component) decreased the level of methylation of the first five CpGs, leading to an increase in Nrf2 expression(193). In this study, hypermethylation of the first 5 CpGs of the Nrf2 promoter was identified in JB6 P+ cells, leading to reduced Nrf2 activity. The epigenetic silencing could be attributed to the epigenetic alterations, e.g., overexpression of HDACs or DNMTs, because these epigenetic modification enzymes were found to be highly expressed in the control cells.

A previous study showed that Nrf2 is involved in the cytoprotective effects of tanshinone IIA by reducing intracellular redox status in human aortic smooth muscle cells and protecting against oxidative stress via the ERK and PKB signaling pathways(194). However, the exact mechanisms by which TIIA regulates Nrf2 have not been identified. Our present study shows for the first time that TIIA epigenetically regulates Nrf2 activation by decreasing promoter DNA methylation. This is associated with the fact that TIIA inhibits the expression of DNMTs (Figure 3.6). Although DNMT activity was not affected by TIIA at the cellular level (Figure 3.7), the enzymatic assay of DNMTs included all of the subtypes of DNMTs, and the results reflected a combination activity of all subtypes but not a specific DNMT which might be crucial for Nrf2 promoter methylation. In addition, it has been reported that cellular DNMT level can be affected at both transcription and post-translation levels (195-197), which could lead to the change of gene methylation profile. Thus, it is possible that TIIA alters Nrf2 promoter methylation pattern when total

DNMTs activity remains unchanged. On the other hand, DNA methylation has been shown to have crosstalk with histone modifications at multiple levels. In this study, our results show that the expression of HDAC1, 3, 4 and 8, as well as HDAC enzyme activity, were significantly decreased in a dose-dependent manner by TIIA. To our surprise, in spite of slightly inducing open histone marker acetyl-H3 at global level, TIIA treatment did not increase the enrichment of acetyl-H3 to the promoter region of the Nrf2 gene where the first 5 CpG locates. Nevertheless, TIIA treatment enriched the recruitment of RNA polymerase II complex on transcription start site of Nrf2 gene. These findings suggest that TIIA does not directly affect histone structure modification associated with the enhanced Nrf2 transcription activity. Considering some HDACs could involve in DNMT complex that mediate DNA methylation (198), inhibition of HDACs by TIIA may also indirectly contribute to the de-methylation effects of TIIA on Nrf2 promoter. Thus, alteration of the expression of some specific DNMTs and HDACs by TIIA would contribute to its capability of epigenetic modulation.

Danshen has been used in China in patients with cardiovascular diseases for many years, and it is approved by the State Food and Drug Administration of China. Our studies show that TIIA inhibited the transformation of JB6 P+ cells at doses of 5–10 μM (Figure 3.2). Previous studies indicated that the blood levels of tanshinones were less than 1 μM after oral administration. This may raise concerns about the in vitro and in vivo relevance because the blood level is far below the in vitro effective concentration level. However, because Danshen has been widely used with a variety of dosage forms and the safety and toxicity of Danshen in human subjects are well known, it is feasible to optimize the formulation to achieve a high circulating concentration in the human body. In addition,

because other components in Danshen have not been found to be effective, the development of TIIA as a pure component is highly feasible. Tanshinone I, another active component in Danshen, has similar structure to TIIA. It has been reported that Tanshinone I is a potent Keap1-C151-dependent Nrf2 activator which can stabilize Nrf2 by hindering its ubiquitination(199). Although both compounds are able to upregulate Nrf2 pathway, the underlying mechanisms may be different. Keap1 was found to be involved in Nrf2 regulation by Tanshinone I (199), and epigenetic modulation could also play a major role and potentially be the main mechanism contributing to the upregulation of Nrf2 by TIIA.

3.5 Conclusion

In conclusion, the present work discovered that Tanshinone IIA significantly inhibits HDACs (HDAC1, 3, 4 and 8) and DNMTs (DNMT1, 3a and 3b) at both the mRNA and protein levels. The inhibitory effect of these epigenetic modification enzymes induced Nrf2 activity via demethylation of the Nrf2 gene promoter in JB6 P+ cells, indicating a potential role in chemoprevention of skin cancer.

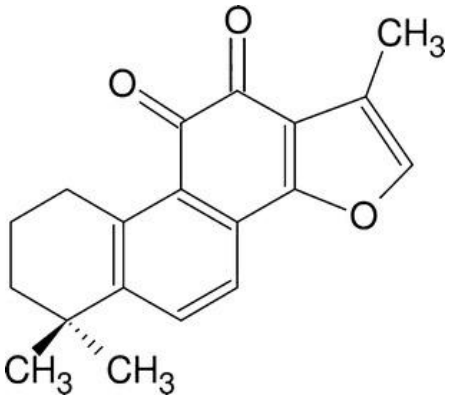

Figure 3.1 Chemical structure of Tanshinone IIA.

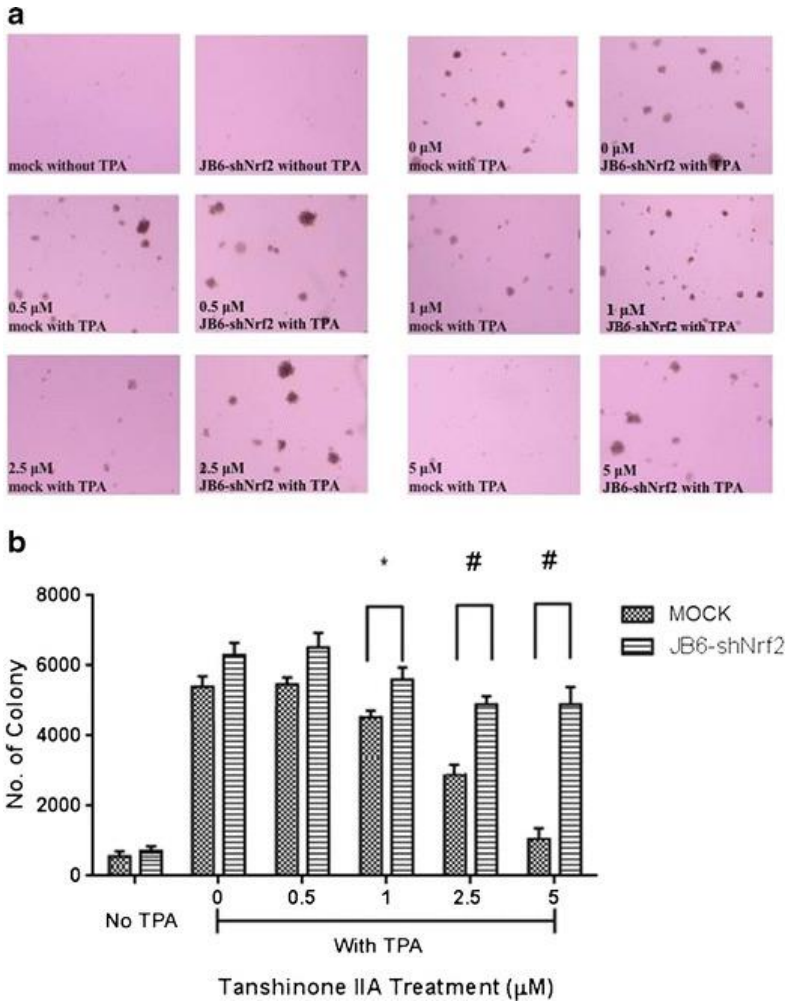

Figure 3.2 Inhibitory effect of TIIA pre-treatment on the TPA-induced transformation of shMock and shNrf2-transfected JB6 P+ cells. Cells were treated with TIIA (0-5.0 μM) for five days. The TIIA pre-treated cells (at a density of 8,000 cells/well) were then transferred to soft agar containing TPA in 6-well plates for an additional fourteen days. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. (A) Representative images of each group under microscope; (B) Graphical data are presented as the average of triplicate results from two independent experiments. *P<0.05; #P < 0.01, indicating a significant increase in colony formation compared with the shMock JB6 P+ cells in each treatment group.

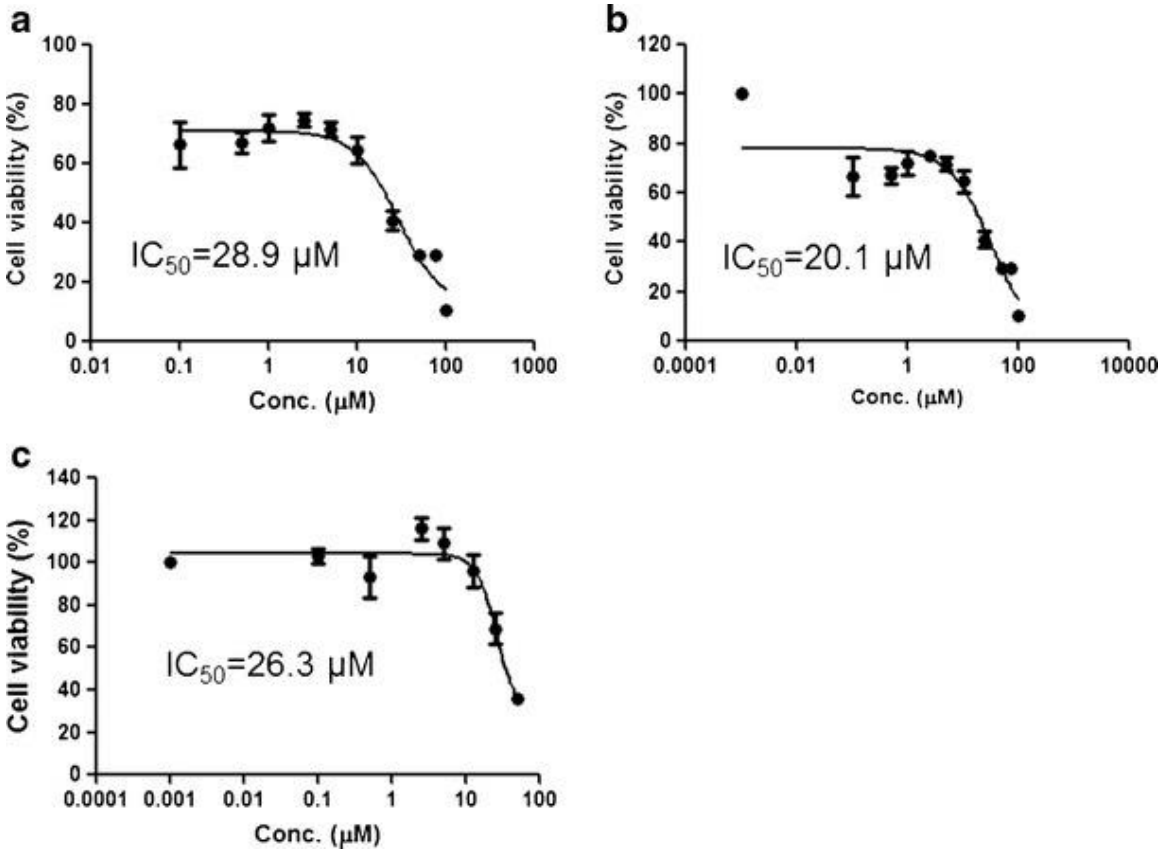

Figure 3.3 Cell viability of JB6 P+, JB6-shNrf2 and HepG2-C8 cells after treatment by TIIA for 24 hours. (A) JB6 P+; (B) JB6-shNrf2; (C) HepG2-C8. The IC50 values were calculated using Emax sigmoid method.

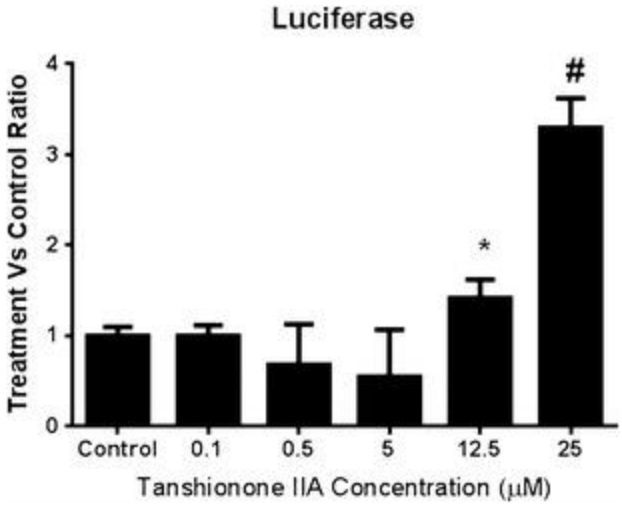

Figure 3.4 Induction of ARE-luciferase activity by the treatment of TIIA with concentrations from 0-25 μM in HepG2-C8 cells expressed with ARE-luciferase vector. The normalization of the luciferase activity was performed based on protein concentrations, which were determined using a BCA protein assay. The data were obtained from three independent experiments and expressed as the inducible fold change compared with the vehicle control. *and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences between the treatment and control group.

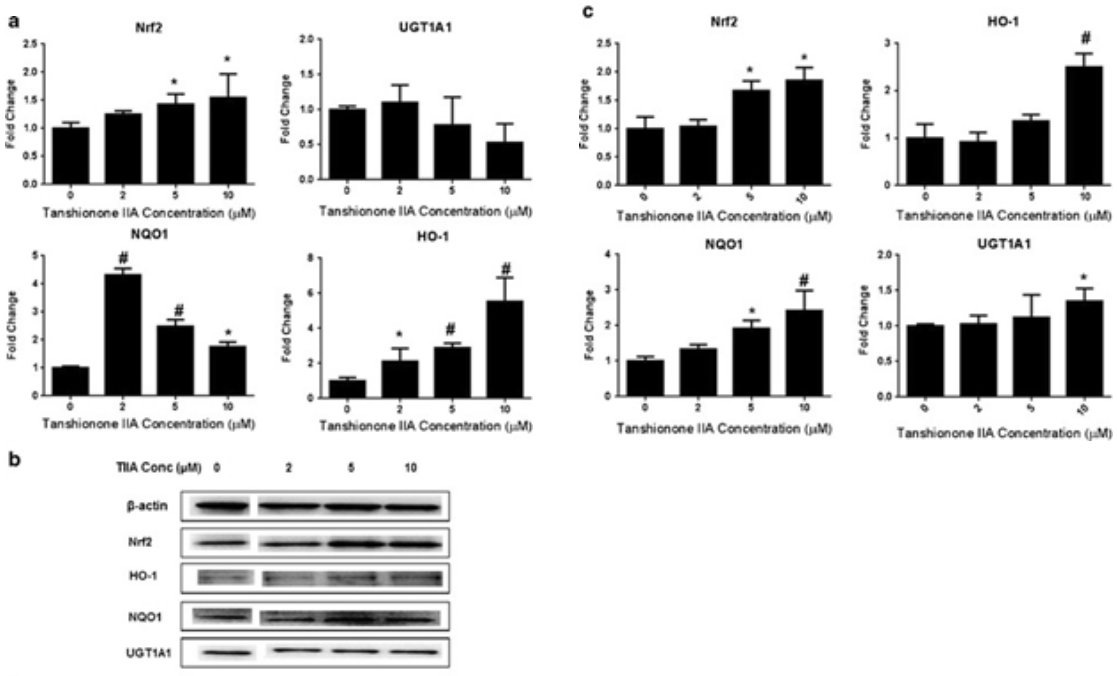

Figure 3.5 Effect of TIIA on Nrf2 mRNA and protein expression of Nrf2 target genes (HO-1, NQO1 and UGT1A1) in JB6 P+ cells. Cells were incubated with various concentrations of TIIA (2-10 μM) for five days. A: TIIA increased the mRNA levels of Nrf2 and its downstream representative enzymes including HO-1, NQO1, and UGT1A1; B: Western blot images of Nrf2 and its downstream genes including HO-1, NQO1, and UGT1A1; C: TIIA (2-10 μM) increased the protein expression of Nrf2 and Nrf2 downstream enzymes. The graphical data are presented as the mean ± SD from three independent experiments. *and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each target protein or mRNA compared with its level in non-TIIA-treated cells.

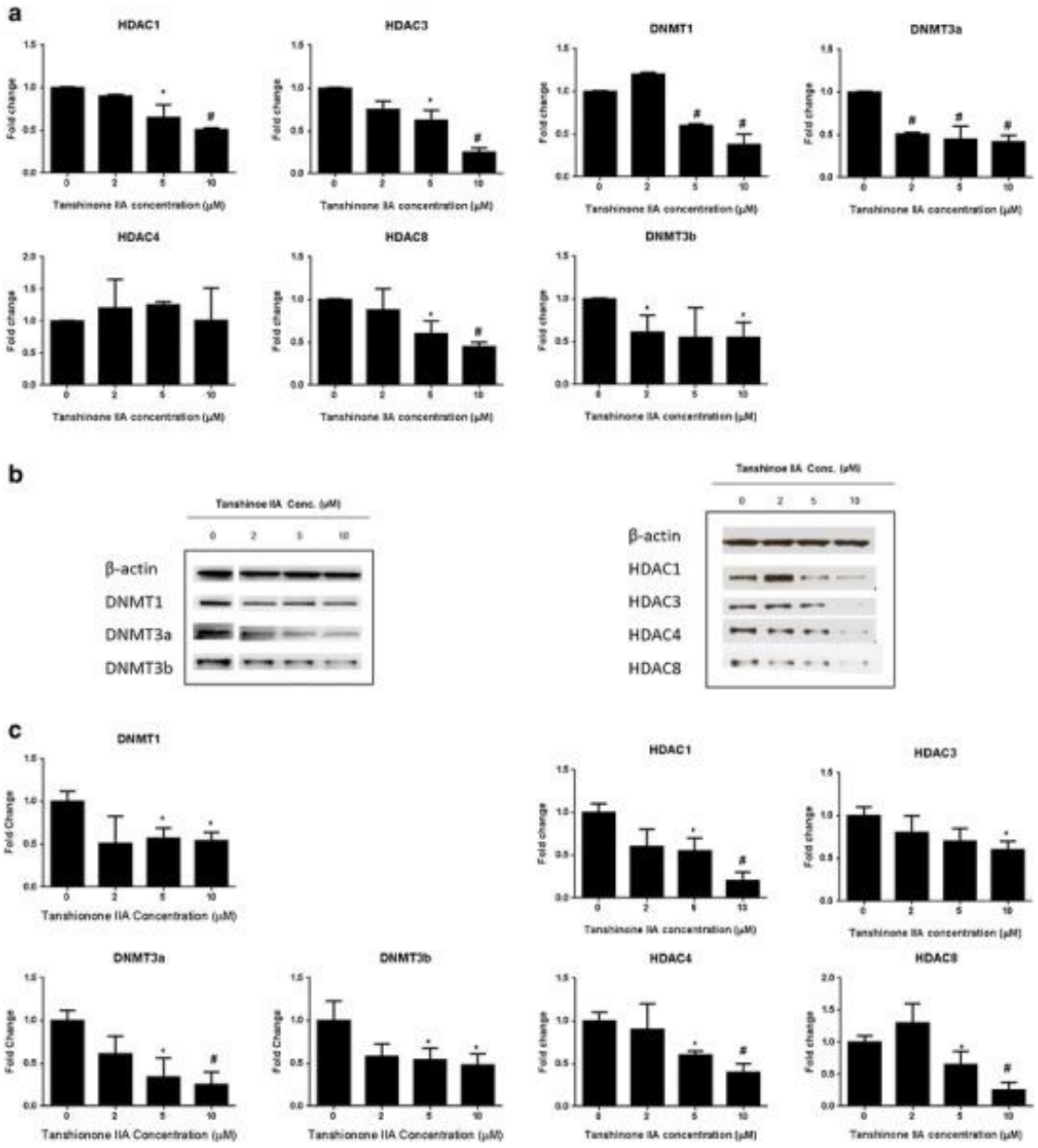

Figure 3.6 Effect of TIIA (2-10 μM) on DNMT and HDAC mRNA and protein expression in JB6 P+ cells. Cells were incubated with various concentrations of TIIA (2-10 μM) for five days. The expression of DNMT1, 3a and 3b as well as HDAC1, 3, 4, and 8 mRNA and proteins was detected by real time PCR and western blotting respectively. A, TIIA decreased the mRNA level of DNMT1, 3a, and 3b, and HDAC1, 3, and 8. B. Western blot images of DNMTs including DNMT1, 3a, and 3b, as well as HDAC1, 3, 4, and 8. C, TIIA

significant inhibit the protein levels of DNMT1, 3a, and 3b, as well as HDAC1, 3, 4, and 8; The graphical data are represented as the mean ± SD from three independent experiments. *and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each group compared with its level in non-TIIA-treated cells.

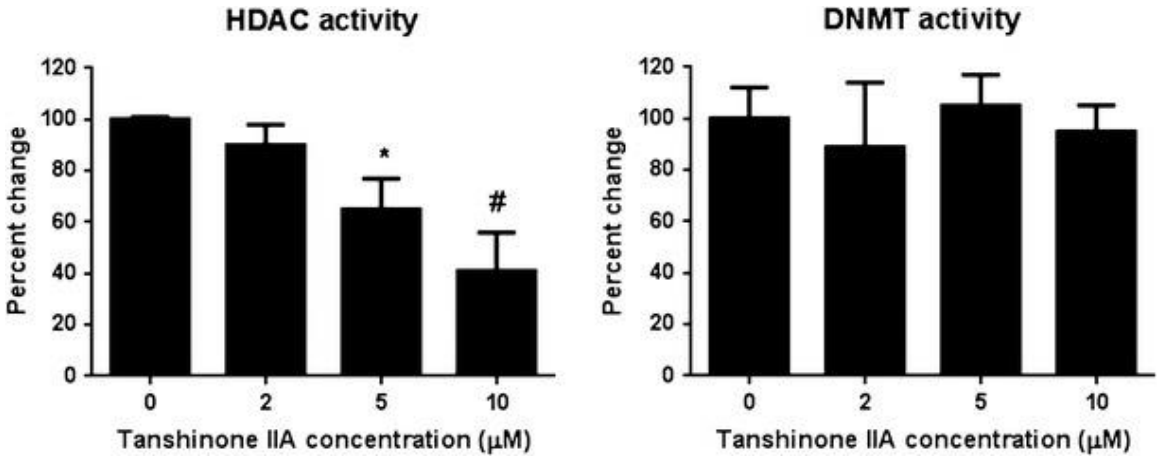

Figure 3.7 The relative HDAC and DNMT activities after TIIA treatment. HDAC and DNMT activity assay was performed using EpiQuik Nuclear Extraction Kit. The relative HDAC activity was calculated based on the ratio of the HDAC activity of the TIIA treatment group to that of the control group. TIIA significantly inhibited the relative HDAC activity by 50%. The graphical data are represented as the mean ± SD from three independent experiments. *and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each group compared with its level in non-TIIA-treated cells.

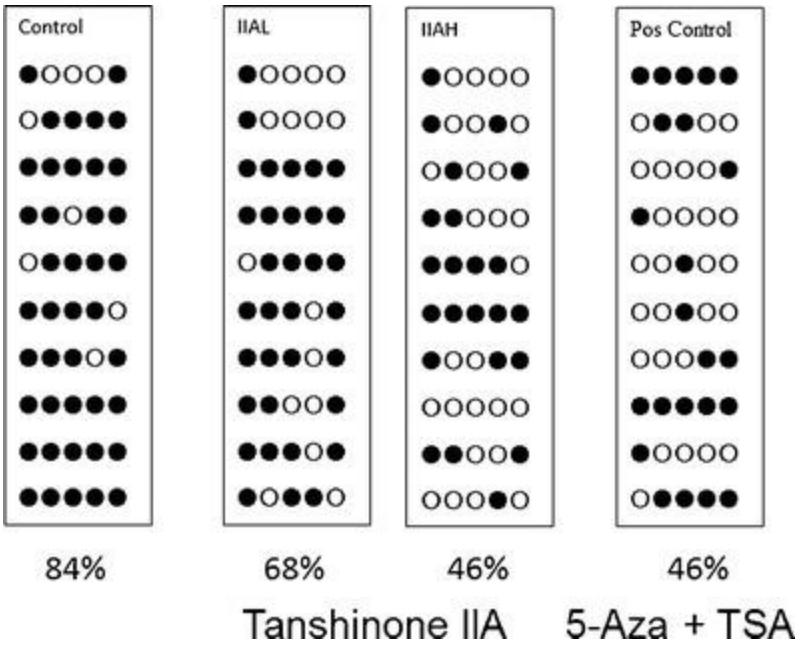

Figure 3.8 Effect of TIIA on methylation alteration of the Nrf2 promoter regions in JB6 P+ cells. A, Genomic DNA from non-treatment cells; B, Genomic DNA was extracted from TIIA-treated cells after five days of treatment; C, the 5-aza (500 nM)/TSA (100 nM) combination treatment. The filled and open dots indicate methylated and unmethylated CpGs, respectively. Methylated CpG ratio: the percentage of methylated CpGs was based on the total CpGs in each treatment group. IIAH: TIIA high concentration (10 μM); IIAL; TIIA low concentration (5 μM); Pos Control: positive control (5-aza (500 nM) + TSA (100 nM)).

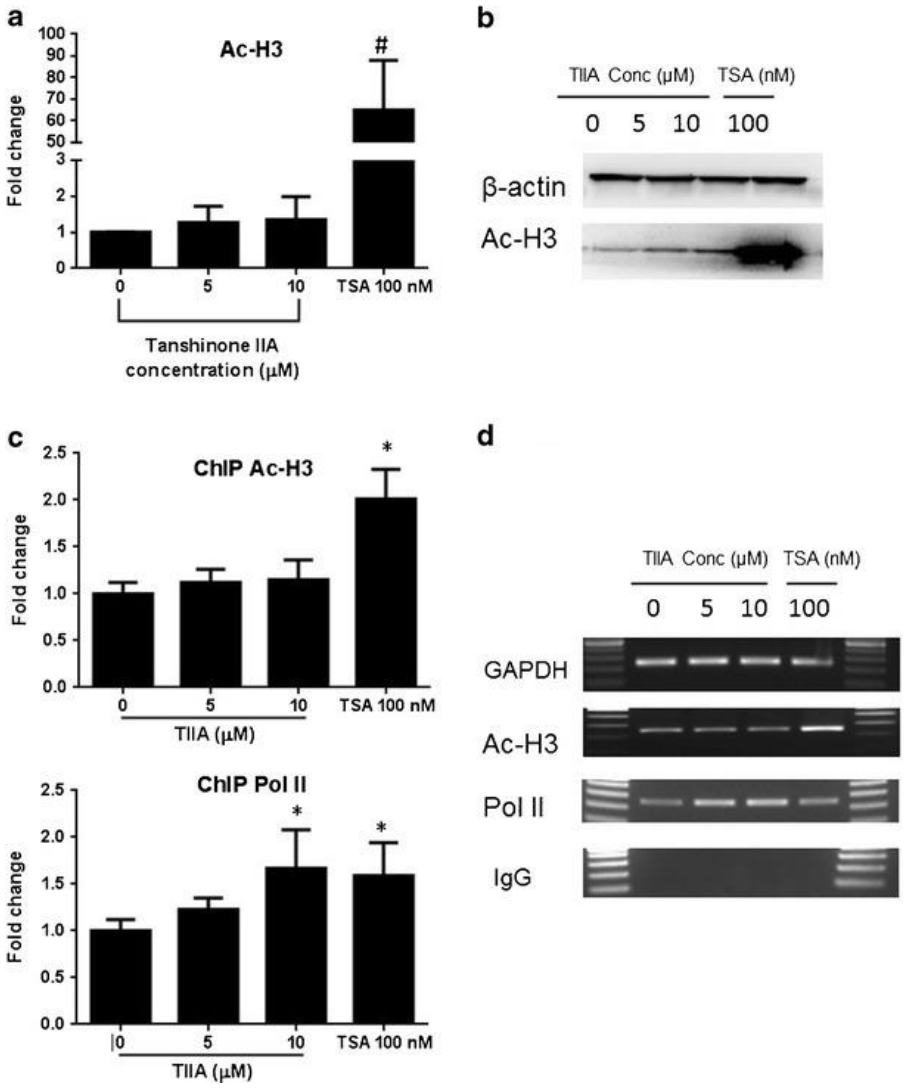

Figure 3.9 Effect of TIIA on histone modification associated to Nrf2 gene promoter. Cells were incubated with TIIA (5 and 10 μM) for five days. The total level of Ac-H3 was detected by western blotting. A. TIIA increased the protein level of Ac-H3. B. Western blot images; ChIP assays were performed to analyze the enrichment of DNA binding to Ac-H3 and Pol II using primers cover the first 5 CpGs or TSS of Nrf2 gene promoter, respectively. (C) The enrichment of the Ac-H3 and Pol II binding DNA was determined by PCR. Relative ratio was calculated using ImageJ software and present by the fold change compared to control group. Data are expressed as mean ± SD from three

independent experiments. (D) Regular PCR was performed to compare the immunoprecipitated DNA versus control group, a representative result is shown from three independent experiments. Trichostatin A (TSA) was used as positive control. *and # represent $P < 0.05$ and $P < 0.01$, respectively.

Chapter 4 Epigenetic Blockade of Neoplastic Transformation by Bromodomain and Extra-Terminal (BET) Domain Protein Inhibitor JQ-1[10,11,12]

4.1 Introduction

Accumulating experimental evidence has suggested that epigenetic alterations are involved in cancer development. DNA methylation on 5-cytosine (5-mC) and covalent modifications to histone tails are the major mechanisms that maintain DNA stability and control gene transcription at the epigenetic level. Recent publications from our group and others have identified that aberrant DNA methylation is associated with altered gene expression in cancer models *in vitro* and *in vivo* (200, 201). In addition, loss of 5-hydroxymethylcytosine (5-hmC) and ten-eleven translocation 2 (TET2) protein expression has been reported in malignant melanoma (202). In addition, chromatin factors such as histone methyltransferases also play a role in tumorigenesis (203, 204). Unlike genetic mutations, epigenetic alterations are heritable changes in gene expression not caused by alterations in the underlying DNA sequences; thus, they are potentially reversible by chemicals. Therefore, targeting epigenetics for cancer chemopreventive and therapeutic strategies is gaining growing interest. Among all of the epigenetic regulators, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) have been most

[10] Part of this chapter has been published in Biochem Pharmacol. 2016 Oct 1;117:35-45
[11] **Keywords**: Bromodomain; Brd4; JQ-1; neoplastic transformation; NF-κB
[12] **Abbreviations**: BrD, bromodomain; BET, BrD and extraterminal domain; CRE, cAMP response element; CBP, cAMP response element-binding proteins, siRNA, small interfering RNA.

extensively studied, and their inhibitors are on the market for the treatment of myelodysplastic syndromes and cutaneous T-cell lymphoma (205), respectively.

In addition to epigenetic writers such as DNMTs and HDACs, epigenetic readers refer to another group of proteins that recognize the covalent modifications on DNA or histones and perform key roles at the interface between epigenetic memory, chromatin remodeling and transcriptional regulation. The N-acetylation of lysine residues on histone tails is associated with an open chromatin structure and transcriptional activation (206). More recent studies have demonstrated that the molecular recognition of acetyl-lysine is principally mediated by bromodomains (BrDs) (207). In humans, there are estimated to be 46 different BrD-containing proteins that encode a total of 61 BrDs (208). Despite large sequence diversities, all BrD-containing proteins share an evolutionarily conserved left-handed four-helix bundle, where the N-acetyl lysine is recognized and anchored by a hydrogen bond with an asparagine residue in a central hydrophobic cavity. This module is linked by diverse loop regions that discriminate between different acetylated lysine residues and determine substrate specificity. The BrD and extraterminal domain (BET) family consists of BRD2, BRD3, BRD4 and BRDT, which share a common domain architecture characterized by two amino-terminal BrDs. BET proteins recognize acetylated lysine residues on histone tails, and recruit chromatin-modifying enzymes, transcription factors, transcriptional co-activators and transcriptional co-repressors, thereby coupling histone acetylation and gene expression (209).

Recent studies have built a compelling rationale for targeting BET proteins in cancer because of their critical roles in tissue development, inflammatory responses and tumor progression (209). For example, high BRD2 levels have been found in human

leukemic cell lines and primary leukemic blasts, whereas lymphoid-restricted constitutive expression of BRD2 leads to the development of aggressive B-cell lymphoma in mice (210). Fusions of BRD4 or BRD3 with nuclear protein in testis (NUT) that generate an in-frame chimeric peptide with tandem N-terminal BrDs preceding the NUT protein result in a proliferation advantage and genetically define an aggressive form of human squamous cell carcinoma as NUT midline carcinoma (NMC) (211). In addition, siRNA silencing of the BRD–NUT oncoproteins in NMC cell lines arrests the cell cycle and prompts squamous differentiation.

Subsequently, small-molecules with high potency and specificity toward BET proteins have been developed, including JQ-1, I-BET151, MS417 and PFI-1 (44, 212, 213). These compounds competitively bind to acetyl-lysine recognition motifs, inhibit the recruitment of BET proteins to chromatin and repress downstream transcriptional programs (212). In particular, inhibition of BET has been introduced as a novel strategy to target oncogenic c-Myc (214, 215), the transcription of which is associated with locally and globally enhanced acetylation of the histone lysine side chain (216, 217). Furthermore, BET inhibitors have shown promising efficacy against solid tumor progression in recent reports, including lung adenocarcinoma, melanoma and hematological malignancies (218-220). However, the role of BET proteins in the early stages of carcinogenesis has not yet been studied.

Tumor formation is a long-term process that typically comprises three different stages: initiation, promotion and progression (221, 222). In this study, we investigated the anti-tumor activity of the Brd4 inhibitor JQ-1 with an emphasis on tumor initiation. Neoplastic transformation of cells is one of the major processes during early carcinogenic

transformation. We assessed the efficacy of pharmacologically inhibiting BET proteins using the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced JB6 P+ cell transformation model, and its underlying molecular events.

4.2 Materials and Methods

4.2.1 Materials

(±)JQ-1 was generously provided by the Structural Genomics Consortium (Toronto, ON). As manufactured, the compound comes as an enantiomer, and only (+)-JQ-1 is the active form. Bacteriological agar, Eagle's basal medium (BME), and TPA were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), and trypsin-EDTA solution were obtained from Gibco Laboratories (Grand Island, NY).

4.2.2 Cell culture and treatment

JB6 P+ (JB6 Cl 41-5a, ATCC CRL-2010) cells from American Type Culture Collection were maintained in MEM containing 5% FBS at 37°C in a humidified 5% CO_2 atmosphere. HaCaT (human keratinocyte), HCT116 cells were grown in DMEM supplemented with 10% FBS. The cells were grown to 60–80% confluency and seeded at a density of 1×10^5 cells/well in 6-well plates. After 24 h incubation, cells were treated with medium containing various concentrations of JQ-1 for 4 h. After 4 h of JQ-1 treatment, TPA was added to the indicated groups at a final concentration of 20 ng/ml. Next, cells were harvested 8 h after TPA treatment for mRNA extraction and 24 h after TPA treatment for western blotting. HCT116 cells were treated only with the indicated concentrations of JQ-1. DMSO (0.1%) was used as a vehicle control for all experiments.

4.2.3 Anchorage-independent cell transformation assay

Anchorage-independent cell transformation assay was carried out as previously described (102). Briefly, Eagle's basal medium agar (3 ml of 0.5%) containing 10% FBS with or without JQ-1 was layered onto each well of the 6-well plates. JB6 P+ cells were treated with various concentrations of JQ-1 for 4 h; then, 8×10^3 pretreated cells were mixed with 1 ml of 0.33% Eagle's basal medium agar and seeded on the top of the 0.5% agar layer. The cells were maintained with 0.1% DMSO (vehicle control), TPA (20 ng/ml) alone or a combination of TPA (20 ng/ml) and designated concentrations of JQ-1 in a 5% CO_2 incubator at 37°C for 14 days, at which time colonies were photographed under a light microscope using the Nikon ACT-1 program (Version 2.20; LEAD Technologies) and counted using ImageJ software (Version 1.40g; NIH).

4.2.4 RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using TaqMan Reverse Transcription Reagents (Life Technologies, Grand Island, NY). The mRNA expression of specific genes was determined by quantitative real-time PCR using the first-strand cDNA as the template in the ABI79000HT system.

4.2.5 Western blotting

Whole-cell lysates were prepared from treated cells using RIPA buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Proteins (20 μg from each sample)

were resolved by 4 to 15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and electro-transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 buffer, the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. Finally, the blots were visualized using the SuperSignal enhanced chemiluminescence (ECL) detection system and documented using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). The intensity of the bands was analyzed by densitometry using the ImageJ program (Version 1.40g; NIH).

4.2.6 Cell cycle distribution analysis

Cell cycle distribution analysis was performed using propidium iodide staining method as previously reported (223). HCT116 cells were seeded in 60-mm Petri dishes followed by serum-starvation for 24 h in serum free medium. Next, the cells were treated with complete DMEM containing either 0.1% DMSO (as a negative control) or the indicated concentration of JQ-1 (100 and 500 nM) for another 24 h. Following treatment, cells were harvested by trypsinization and washed twice in ice-cold phosphate buffered saline (PBS). Cells were collected by centrifugation at 500*g for 5 min, and cell pellets were then re-suspended in 300 µl of PBS and fixed by gradually adding 700 µl of ethanol. The fixed cells were then stained in 500 µl of PBS containing 10 µg/ml propidium iodide and 100 µg/ml RNase A at room temperature for 30 min in the dark. The cell cycle distribution was then analyzed by flow cytometry using the FACS/Cell Sorting Core Facility at EOHSI, Rutgers University.

4.2.7 Luciferase reporter activity assay

The genomic sequences of the human COX2 and IL6 promoter regions were retrieved from UCSC gene browser. The targeting sequences were amplified by PCR and cloned into the pCR2.1 TOPO vector (Life Technologies, Grand Island, NY). These sub cloning plasmids were amplified in *E. coli*, isolated and digested with KpnI and XhoI. The designated fragments were then inserted into the luciferase promoter region of pGL4.15 [luc2P/Hygro] vector (Promega, Madison, WI) as the plasmid for luciferase assay. Sequences of all recombinant plasmids were verified by sequencing (Genewiz, South Plainfield, NJ). For the luciferase activity assay, HaCaT or HEK293 cells were transfected with 400 ng of the indicated reporter plasmids using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's manual. The cells were co-transfected with 100 ng of pGL4.75 [hRluc/CMV] plasmid (Promega, Madison, WI), which constitutively expresses Renilla luciferase as an internal control. The transfected cells were treated with 20 ng/ml TPA alone or in combination with 500 nM JQ-1 for 24 h and were then lysed in dual luciferase lysis buffer. The luciferase activity of each sample was measured using a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The firefly luciferase activities were normalized with corresponding Renilla luciferase activities, and results were presented as the induced fold change compared with the vehicle control.

4.2.8 RNA interference

RNAi duplex oligos (siRNA) targeting murine p300 were designed and synthesized from Integrated DNA Technologies (Coralville, IA). Transfection of siRNA was carried out using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the

manufacturer's instructions. Briefly, 5 μl of si-p300 stock solution (10 μM) in 125 μl of Opti-MEM was added to another 125 μl of Opti-MEM containing 5 μl of Lipofectamine 3000. Universal negative control siRNA was similarly prepared and used as siControl. After 15 min incubation at room temperature, the transfection mixtures were applied to JB6 P+ cells for 24 h in a 37°C incubator. Then, the cells were subjected to further treatments of 20 ng/ml TPA alone or in combination of 500 nM JQ-1.

4.2.9 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted using ChIP Kit - One Step (Abcam, Cambridge, MA) following the manufacturer's instructions. Briefly, JB6 P+ cells were treated with 20 ng/ml TPA for 4 h in the presence or absence of 500 nM JQ-1 pretreatment. After washing twice with PBS, chromatin was cross-linked with 1% formaldehyde for 10 min at room temperature. Next, 1.25 M glycine was added to quench the excess formaldehyde. The cells were pelleted by centrifugation, re-suspended in lysis buffer and sonicated to generate 200- to 500-bp DNA fragments. The cross-linked chromatin fragments were subjected to immunoprecipitation with specific antibodies against Ac-H3K27, Histone H3, BRD4 and RNA Polymerase II (Pol II) (Abcam, Cambridge, MA) to capture protein-DNA complexes. After precipitation, 2 μl of each purified DNA was used as a template for qPCR quantification. Before immunoprecipitation, a small proportion was kept from each chromatin sample as input. These input samples were treated in parallel as the precipitated samples expect for that no antibody was added in the immunoprecipitation procedure. Purified input DNA were geometrically diluted into a series of concentration then used for standard curve in qPCR. The relative enrichment was calculated by locating the precipitated DNA sample on its self-input standard curve.

Then the relative enrichment was normalized to the vehicle control group as fold change. For Ac-H3K27 enrichment quantification, additional normalization to the Histone H3 C-terminal enrichment of each sample was done prior to the comparison with the control group. Non-specific IgG was used as a control to verify the specificity of the ChIP assays.

4.2.10 Statistical analysis

The data are presented as the mean ± SD. The statistical analyses were performed using Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant differences among the means were set at *$P < 0.05$, **$P < 0.01$, and #$P < 0.001$.

4.3 Results

4.3.1 JQ-1 inhibits TPA-induced JB6 P+ cell transformation

JQ-1 treatment was tested on the TPA-induced anchorage-independent growth of JB P+ cells in the soft agar assay. As shown in Figure 4.1, JB6 P+ cell by itself cannot form colonies in soft agar (anchorage-independent grow condition) unless stimulated with TPA. Both colony number and size were markedly decreased by JQ-1 treatment. Compared with the TPA-treated group, 100 or 500 nM JQ-1 treatment decreased the mean number of colonies by approximately 61% ($P < 0.05$) and 74% ($P < 0.01$), respectively. These results show that JQ-1 significantly suppressed the TPA-induced colony formation of JB6 P+ cells.

4.3.2 BET inhibition decreases TPA-induced oncogenic signaling

To investigate the cellular processes affected by BET inhibition, we analyzed a series of cell cycle and proliferation markers in cells treated with JQ-1 in the context of TPA stimulation. Consistent with previous reports, TPA triggered the phosphorylation of

the mitogen-activated protein kinases (MAPKs) ERK1/2 (Figure 4.2A). Accordingly, elevated levels of anti-apoptotic and pro-growth oncogenes such as Bcl2, Cyclin D1, and c-Myc were observed in response to 20 ng/ml TPA treatment (Figure 4.2B&C). Since JB6 P+ cell line is derived from mouse epidermis and skin cancer is the most commonly diagnosed cancer in the United States, we also evaluated the translational potential of these results on human keratinocyte HaCaT cell line (Figure 4.2D&E). In accordance with the anchorage-independent cell growth assay, JQ-1 treatment reduced TPA-induced Bcl2 expression in both JB6 P+ and HaCaT cells (Figure 4.2C&E). Furthermore, TPA-stimulated Cyclin D1 and c-Myc expression was decreased at both the mRNA and protein levels in a dose-dependent manner in response to JQ-1. Surprisingly, JQ-1 also attenuated the levels of phosphorylated ERK1/2.

4.3.3 JQ-1 induces G1 cell cycle arrest in HCT-116 cells

It has been reported that BRD4 influences mitotic progression by binding to the transcriptional start sites of genes expressed during cell cycle transition (207), which is in agreement with our results above. In order to evaluate whether there exists a common set of transcriptional events mediated by BET in different stage of cancers, such as the cell cycle regulatory genes, we next examined the effect of JQ-1 on cell cycle regulatory genes in a cancer cell line HCT-116. The cells were treated with the indicated concentrations of JQ-1 for 24 h. As shown in Figure 4.3A&B, the cells treated with 100 or 500 nM JQ-1 showed a remarkable decrease in c-Myc, Cyclin A, and Cyclin D1 expression at both the mRNA and protein levels. Cell cycle distribution was then analyzed by flow cytometry after JQ-1 treatment. Accordingly, the results showed a significant increase in the percentage of the cells in G1 phase from 47.7% (0μM) to 70.7% (500 nM) (Figure 4.3C).

4.3.4 JQ-1 alters the expression of HDACs in HCT116 cells

Other epigenetic-modifying compounds such HDAC inhibitors (HDACis) also cause cycle arrest in cancer cells. Interestingly, a recently study revealed the similarity of gene expression alterations induced by BET inhibitors (BETis) and HDACis in murine lymphoma cells (224). Therefore, we investigated a potential interaction between these two levels of epigenetic regulation by performing western blotting to evaluate the effect of JQ-1 treatment on the expression of HDACs. We found that the protein levels of HDAC2, HDAC3, and HDAC4 were significantly reduced in HCT116 cells after JQ-1 treatment, whereas there were no changes in the expression of HDAC1 (Figure 4.4).

4.3.5 JQ-1 down-regulates the mRNA and protein levels of NF-κB target genes

Activation of NF-κB signaling is an important mechanism implicated in tumor progression that is promoted by TPA (225). In our study, 20 ng/ml TPA treatment increased the mRNA expression of NF-κB target genes in both JB6 P+ and HaCaT cells. Compared with the TPA-alone group, cells treated with JQ-1 at concentrations of 100 and 500 nM showed significantly decreased transcription activities of these TPA-induced genes , including COX2 and IL6 (Figure 4.5A&C). The protein level of COX2 was further determined in cells treated with TPA alone or in combination with JQ-1 by western blotting. Corresponding to the qPCR results, the protein expression of COX2 was up-regulated by TPA, whereas JQ-1 reduced the TPA-boosted levels of COX2 (Figure 4.5B&D). These results indicate that the inhibition of Brd4 may block the activation of NF-κB signaling by TPA.

4.3.6 JQ-1 inhibits CRE-associated transcription in luciferase reporter activity

To evaluate how JQ-1 affects the transcription activities of NF-κB target genes, luciferase reporters driven by different regions of the COX2 and IL6 promoters were constructed as shown in Fig. 6A. In addition to NF-κB binding sites, both the COX2 and IL6 promoters have cAMP response elements (CREs), which are recognized by cAMP response element-binding proteins (CREBs). CREB is a transcription factor that binds to CREB-binding protein (CBP) and its paralog p300, which enhances gene expression by relaxing the chromatin structure through its intrinsic histone acetyltransferase (HAT) activity and recruiting Pol II to the promoter. It has been reported that p300 is widely involved in NF-κB-driven transcription (226, 227); therefore, we established plasmids carrying NF-κB binding sequences alone or both NF-κB and CREB binding elements (Figure 4.6A). After transient transfection into the cells, 20 ng/ml TPA substantially increased the luciferase activities among all four reporter constructs at 20 h. Interestingly, 500 nM JQ-1 could not inhibit the luciferase activities induced by TPA in the cells transfected with plasmids carrying NF-κB binding sites alone (COX2-321 and IL6-105). By contrast, 500 nM JQ-1 abolished the luciferase activities (82% decreases) stimulated by TPA in the cells transfected with the reporter constructs containing both NF-κB and CREB binding sites (Figure 4.6B). The results indicate that inhibition of Brd4 might influence the transcriptional activities of NF-κB target genes by indirectly associating with co-activators rather than by blocking the direct translocation and DNA-binding of NF-κB.

4.3.7 JQ-1 decreases the recruitment of Brd4 and Pol II to the COX2 transcription starting site.

As shown above, it appears to be that JQ-1 inhibits the transcription of NF-κB targeted gene indirectly. Therefore, ChIP assay was employed to further examine the COX2 transcription regulatory mechanism on the scope of histone modification in JB6 P+ cells during TPA-induced neoplastic transformation. As shown in Figure 4.7, 20 ng/ml TPA increased the enrichment of Ac-H3K27 on the COX2 promoter, subsequently recruiting Brd4 and Pol II to the COX2 transcription starting site (TSS). JQ-1 treatment did not change the level of Ac-H3K27 associated with the COX2 promoter but did decrease the TPA-induced recruitment of both Brd4 and Pol II to this region, which is proposed to be essential for transcription initiation and elongation. The specificity of ChIP assays was verified by using non-specific IgG in the precipitation procedure as a negative control, which has no specific amplification in qPCR.

4.3.8 JQ-1 inhibits the p300-dependent transcription activation of COX2

We conducted gene silencing using p300 siRNA to confirm that JQ-1 influences the NF-κB-mediated COX2 gene transcription through histone modifications, rather than interfering with the direct NF-κB-DNA binding. The efficiency of siRNA delivery into cells was confirmed by qPCR and western blotting. The transfection of negative control (NC) siRNA, which has no homology to any known mammalian gene, was performed in parallel with that of p300 siRNA. We found that the knockdown of p300 attenuated the TPA-stimulated enrichment of Ac-H3K27 on the promoter region of COX2, accompanied with reduced Brd4 on TSS (Figure 4.8A). Correspondingly, lower mRNA and protein levels of COX2 were observed in samples from the si-p300 group, compared with the si-

NC group. However, after RNA interference of p300, 500 nM JQ-1 did not show any further inhibitory effects on COX2 induction (Figure 4.8 B&C).

4.4 Discussion

In this study, we found that the inhibition of Brd4 by JQ-1 blocked TPA-induced JB6 P+ cell transformation, indicating a potential role for BET proteins in promoting the early stages of carcinogenesis. JB6 P+ is a promotion-sensitive murine epidermal cell line that undergoes neoplastic transformation after environmental challenges, including TPA, EGF, and UVB (228). It has been demonstrated to be a useful model to study tumor promoter-induced carcinogenic processes at the molecular level (229). As shown in Fig. 2, TPA promoted the transformation of JB6 P+ cells, which was accompanied by elevated p-ERK1/2, Bcl2, Cyclin D1, and c-Myc. Although HaCaT cells are not sensitive to TPA stimulation for anchorage-independent growth, similar molecular alterations were observed in this cell line after TPA and JQ-1 treatment.

Notably, JQ-1 down-regulated the mRNA and protein expression of c-Myc, which is one of the most commonly deregulated oncogenes in human cancers (230, 231). The direct inhibition of c-Myc has not proven successful since the lack of a ligand-binding domain (232). Given that chromatin is a platform for c-Myc signal transduction (233), selective small-molecule inhibitors were developed to inhibit Myc transcription and function through the competitive displacement of chromatin-bound BET proteins. These compounds showed evidence of anti-tumor efficacy in several preclinical models (214, 215, 234-237). Their growth inhibitory effects against cancer cells were mainly attributed to the suppression of c-Myc protein levels as well as the Myc-dependent transcriptional network. In our study, JQ-1 attenuated the TPA-induced expression of anti-apoptotic and pro-

proliferative oncogenes during neoplastic transformation of JB6 P+ cells by disrupting Brd4 activity. An unexpected observation was that JQ-1 treatment reduced the levels of phosphorylated ERK1/2, a finding that was also found in several previous reports. Segura et al. noted that this effect may result from the reduced transcription of ERK1 following BRD4 inhibition in melanoma cells (219). However, a plausible mechanistic explanation remains to be fully elucidated.

In addition to this neoplastic transformation model, we assessed the influence of JQ-1 in cancer cell line HCT116 with a special interest on cell cycle. Similarly, JQ-1 treatment resulted in significant decreases in c-Myc, Cyclin A, and Cyclin D1 expression in HCT116 cells, which are associated with G1 cell cycle arrest (Figure 4.3). These results of our study are consistent with those in human acute myelogenous leukemia (AML) cells (238) and melanoma cells (219) in response to JQ-1 or other BET inhibitors, indicating that a common set of transcriptional events mediated by BET may exist among different stage of cancers.

The combination of small-molecule inhibitors targeting chromatin remodeling enzymes has recently become an attractive strategy that may have lower toxicity and stronger inhibitory effects in several cancer types than using these inhibitors alone (239, 240). Specifically, it has been reported that the HDAC inhibitor SAHA synergizes with JQ-1 to achieve a more potent suppressive effect in advanced pancreatic ductal adenocarcinoma (PDAC) (241). Surprisingly, as mentioned previously, there is an overlap of genes induced by BETis and HDACis. It was hypothesized that the induction of those genes is due to the relocation of the transcription elongation factor p-TEFb to other transcriptional complexes upon BETi and HDACi treatment (224). However, the exact

molecular mechanism has not been elucidated. In our study, we examined the expression of HDACs and found that JQ-1 decreased the protein level of several HDACs, partially explaining the similarity between genes induced by BETis and HDACis.

In addition to alterations of cell cycle- and apoptosis-related genes, activation of NF-κB has been observed in response to a wide variety of extracellular stimuli such as TPA, growth factors, cytokines, and UV radiation (2). Activation of NF-κB has been implicated in the development of skin, colon, and prostate cancer in human clinical cases and mouse models (242-244). Therefore, the inflammation-associated NF-κB signaling pathway is essential in tumor promoter-induced transformation and the development of tumors. Our results also demonstrated that TPA up-regulated the expression of NF-κB target genes such as COX2, IL6 and uPAR in JB6 P+ cells (Figure 4.5), whereas inhibition of Brd4 by JQ-1 reduced TPA-induced inflammatory gene expression. Nicodeme et al. reported that the synthetic compound I-BET (a BET inhibitor) suppressed the inflammatory response in activated macrophages (245). By interfering with the binding of BET proteins to acetylated histones, I-BET disrupts the assembly of the chromatin complexes that are essential for initiating mRNA transcription of inflammatory genes. The common anti-inflammatory effects of JQ-1 and I-BET between our experiments and previous reports indicate that targeting Brd4 may serve as a novel strategy in the regulation of NF-κB downstream gene expression.

To determine how JQ-1 influences the transcription of NF-κB target genes, we performed luciferase reporter assays driven by various fragments from the COX2 and IL6 promoters. The transcriptional activation of NF-κB involves a series of cofactors, particularly CBP/p300 that possesses HAT activity (246). For instance, the induction of

COX2 expression has been shown to require both the NF-κB and CREB binding sites in bronchial airway epithelial cells stimulated with lipopolysaccharide and TPA (247). The activated CREB protein binds to a CRE region and then the KIX domain of CBP/p300, relaxes the chromatin structure and recruits the transcriptional machinery, including Pol II, resulting in the enhanced transcription of target genes (248, 249). In our study, JQ-1 inhibited luciferase expression from reporter constructs carrying both NF-κB and CREB binding sites but had minimal effect on those only containing NF-κB elements. These results suggest that BET proteins may be implicated in the CREB-CBP/p300-mediated transcriptional enhancement of NF-κB target genes. Interestingly, in vascular smooth muscle cells, NF-κB is required for both basal and Angiotensin II (Ang II)-mediated IL6 expression, whereas CREB is required only for Ang II-induced effects (250). This observation suggests the inhibition of BET proteins might have the potential to selectively block pathological alterations of NF-κB target gene expression under stress conditions.

In specific, elevated levels of COX2 have been observed frequently in neoplasms of epithelial origin. In skin cancer, experimental evidence has suggested that high levels of COX2 expression and PGE_2 production play a role in tumorigenesis (251, 252). Therefore, the suppression of prostaglandin synthesis by inhibiting COX2 expression was suggested as a promising chemopreventive strategy (253). Our results showed that JQ-1 suppressed TPA-induced COX2 expression in JB6 P+ cells (Figure 4.5). We further evaluated how JQ-1 treatment regulated the transcription of COX2 by siRNA silencing of p300 and ChIP assays. Interestingly, despite the attenuated level of COX2 induced by TPA, JQ-1 had a negligible effect on COX2 expression in the context of p300 silencing (Figure 4.8B & C). This result is in agreement with the luciferase activity assays described above. A previous

study demonstrated that the intrinsic HAT activity of p300 plays an important role in opening up the chromatin structure and activating COX2 transcription (254). In our study, we found that TPA treatment increased the enrichment of acetyl-H3K27 on the promoter region of the COX2 gene, which is proposed to be specifically mediated by CBP/p300 (255). Correspondingly, our results have shown that p300 siRNA treatment attenuates the TPA-induced Ac-H3K27 enrichment at COX2 promoter, accompanied with the decrease of Brd4 enrichment at gene TSS (Figure 8A). On the other hand, JQ-1 did not change the level of acetyl-H3K27 but decreased the recruitment of Brd4 and Pol II (Figure 7). Given that Brd4 functions as an adaptor that binds to acetylated chromatin and promotes transcriptional elongation, it is possible that JQ-1 blocks COX2 overexpression in JB6 P+ cells during neoplastic transformation underlying a histone modification-related epigenetic mechanism.

4.5 Conclusion

Collectively, we have demonstrated that the inhibition of BET proteins by JQ-1 blocks JB6 P+ cell neoplastic transformation focusing on its potent anti-proliferative and anti-inflammatory effects. Indeed, cancer initiation involves more complicated cellular and molecular oncogenic alterations, even in the same model as the present work (256, 257). It would be helpful to broaden the understanding of pharmacological BET inhibition by exploring the effect on those pathways in the future work. On the other hand, it has been reported that JQ-1 could inhibit other oncogenic signals by regulating the transcriptional events, such as prostate cancer with constitutively AR activation (258) and non-small cell lung cancer driven by Kras mutant (236). In addition, these in vivo studies also have shown JQ-1 is well tolerated in animals at effective doses, which implies a potential for further

clinical investigation. In summary, epigenetic modifications clearly affect cancer initiation and progression. A variety of emerging small molecules that modify the tumor epigenetic landscape have shown promising clinical and preclinical results (259). Our study suggests that BET proteins could be potential targets for chemopreventive or therapeutic strategies for early-stage carcinogenesis.

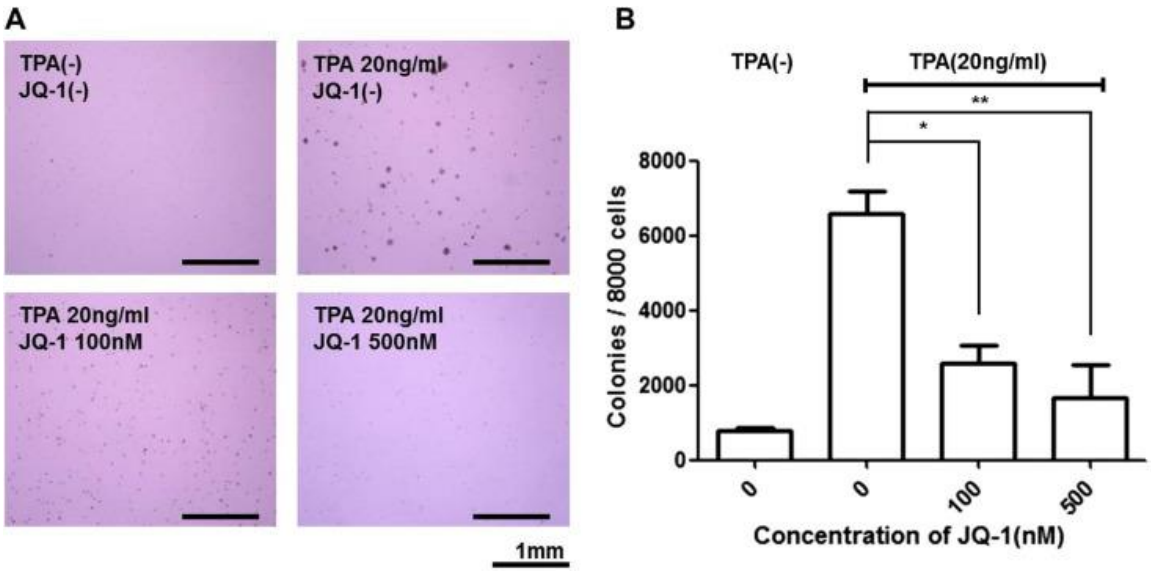

Figure 4.1 Inhibitory effects of JQ-1 on TPA-induced transformation of JB6 P+ cells. Cells were seeded in soft agar containing DMSO (vehicle control), 20ng/mL TPA or a combination of TPA and indicated concentration of JQ-1 in 6-well plates and were allowed to grow for 14 days. The colonies exhibiting anchorage-independent growth were imaged under a microscope, and the colony numbers were counted using ImageJ software. A) Representative images of each treatment group. B) Graphical data of colony numbers in each group are presented as the mean ± SD from three independent experiments. JB6 P+ cells in the vehicle control group rarely formed colonies in agar plate unless treated with TPA. JQ-1 significantly inhibited the TPA-induced anchorage independent growth of JB6 P+ cells. *P<0.05 and **P<0.01 indicate significant differences between the JQ-1-treated group and cells treated with TPA alone.

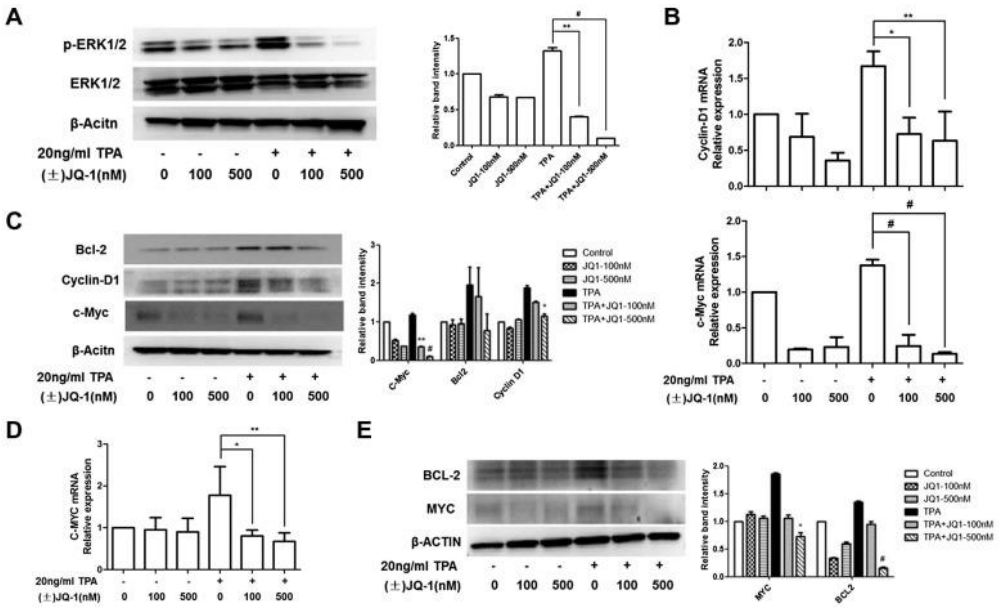

Figure 4.2 Effect of JQ-1 on the mRNA and protein expression of oncogenic genes induced by TPA. JB6 P+ or HaCaT cells were treated with the indicated concentrations of JQ-1 for 4 h, followed by 20 ng/ml TPA treatment. Cells were harvested 8 h post TPA treatment for RNA extraction and 24 h for protein sample preparation. A) JQ-1 attenuated the TPA-induced phosphorylation of ERK1/2 in JB6 P+ cells; band intensity of the blots were digitized and normalized to the vehicle control as fold change, presented as the mean ± SD in the right panel. B) JQ-1 treatment decreased the TPA-induced transcription of Cyclin D1 and c-Myc in JB6 P+ cells. C) Western blotting images of Bcl2, Cyclin D1, and c-Myc in JB6 P+ cells after JQ-1 treatment; right panel shows the bar graph of relative band intensity as the mean ± SD. D) JQ-1 treatment attenuated c-MYC mRNA levels in HaCaT cells. E) JQ-1 treatment decreased c-MYC and Bcl2 protein levels in HaCaT cells; right panel shows the bar graph of relative band intensity. **P*<0.05, ***P*<0.01, and #*P*<0.001 indicate significant differences between the JQ-1-treated group and the cells treated with TPA alone.

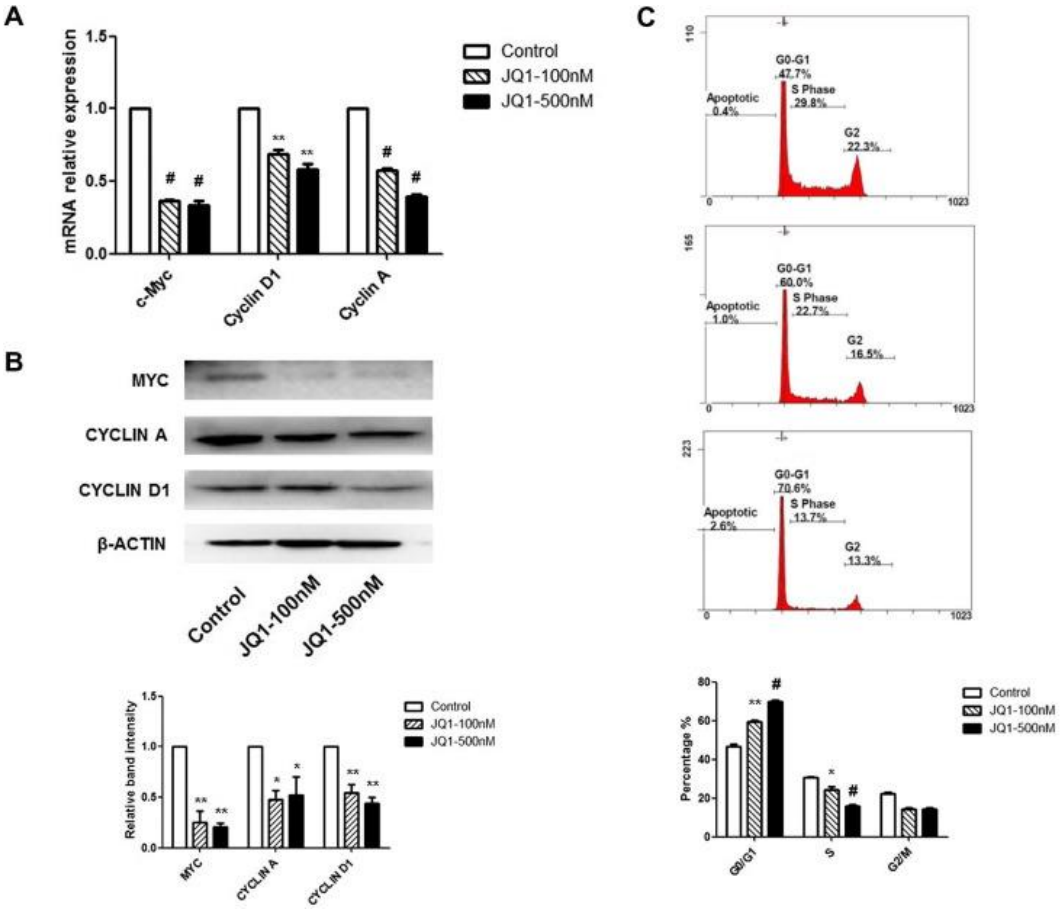

Figure 4.3 Effect of JQ-1 on G1 arrest of HCT-116 cells. HCT116 cells were treated with the indicated concentrations of JQ-1 for 24 h. A, B) JQ-1 suppressed cell cycle-related markers such as c-Myc and Cyclins A and D1 at both the mRNA and protein levels; relative mRNA expression or blot band intensity are presented as the mean ± SD; C) JQ-1 significantly induced G1 cell cycle arrest in HCT116 cells. Representative results from three independent experiments are provided in the upper panel whereas the lower panel shows the cell percentage in each phase as the mean ± SD. The statistical analyses were performed using one-way ANOVA with post-hoc Dunnett's test. *P<0.05, **P<0.01, and #P<0.001 indicate significant differences between the JQ-1-treated group and the vehicle control.

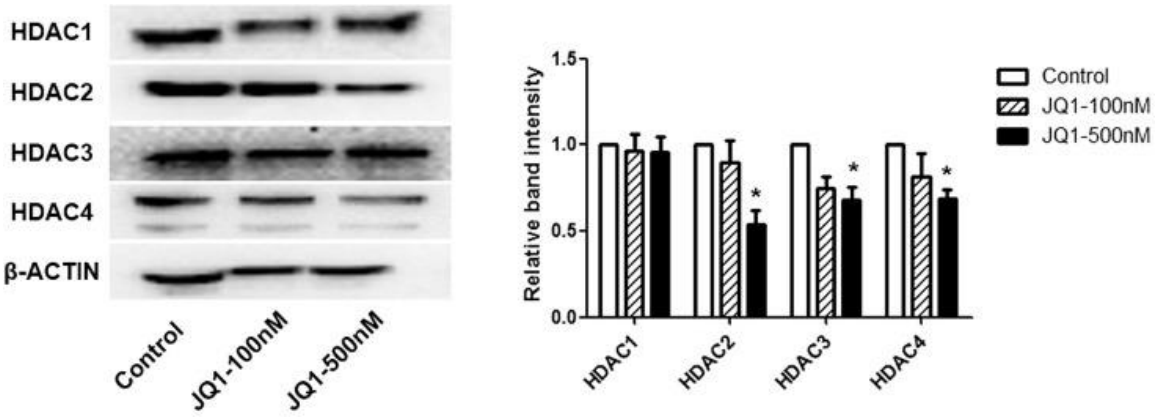

Figure 4.4 JQ-1 altered the protein expression of HDACs in HCT116 cells. The protein expression of HDAC1–4 was determined by western blotting. The relative band intensity (fold change) was calculated by normalizing the intensity of each sample to the vehicle control. Representative bands are shown in the left panel, whereas the bar chart in the right panel presents the mean ± SD of three independent experiments. *P<0.05 indicates significant differences between the JQ-1-treated group and the vehicle control.

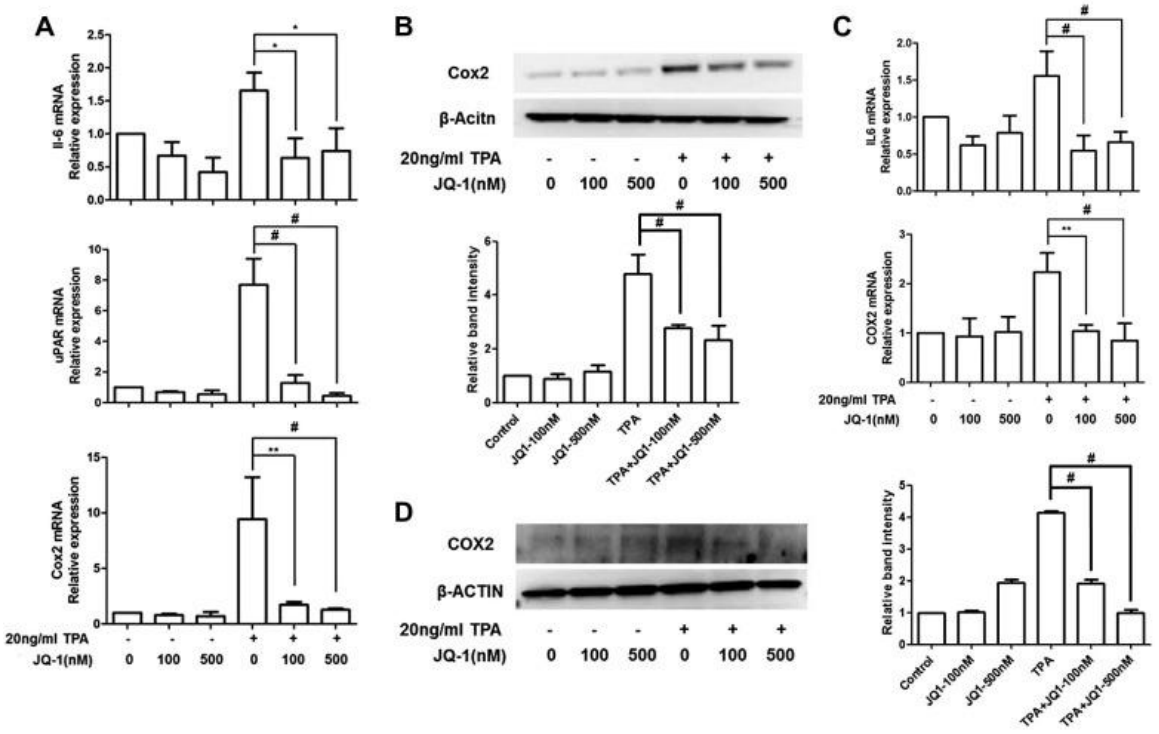

Figure 4.5 Effect of JQ-1 on the activation of NF-κB target genes triggered by TPA. A) JQ-1 treatment decreased the TPA-induced transcription of IL6, uPAR, and COX2 in JB6 P+ cells. B) Western blot images of COX2 expression in JB6 P+ cells after JQ-1 treatment. C) JQ-1 treatment attenuated IL6 and COX2 mRNA levels in HaCaT cells. D) JQ-1 treatment decreased COX2 protein levels in HaCaT cells. *P<0.05, **P<0.01, and #P<0.001 indicate significant differences between the JQ-1-treated group and cells treated with TPA alone.

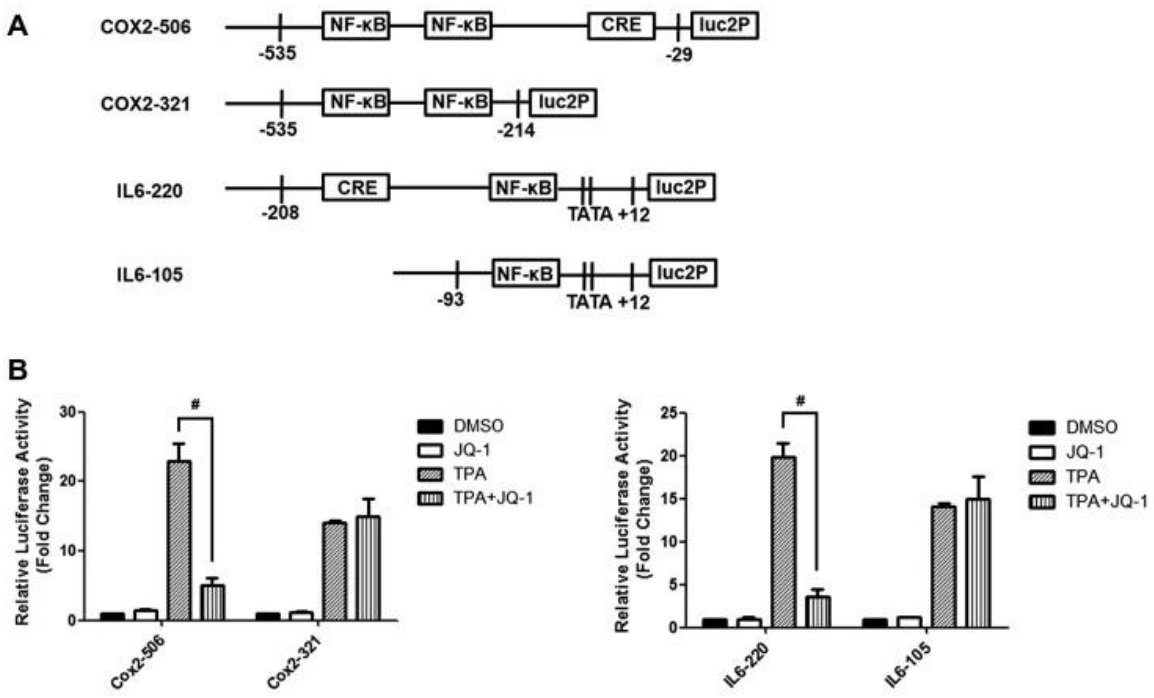

Figure 4.6 JQ-1 is not a direct inhibitor of NF-κB. A) Different constructs of luciferase reporter genes. B) HaCaT cells were co-transfected with 400 ng of the designated pGL4.15 firefly luciferase reporters and 100 ng of pGL4.75 [hRluc/CMV] Renilla luciferase plasmids as an internal control. The transfected cells were treated with DMSO, 20 ng/ml TPA, 500 nM JQ-1, or the combination for 20 h. The firefly luciferase readings were normalized to the Renilla luciferase reading first; then, the fold changes were calculated by comparing each treatment group to the vehicle control. JQ-1 inhibits the luciferase activity in the cells transfected with the plasmids carrying CRE elements (*#P*<0.001). No significant changes were observed after JQ-1 treatment in the cells transfected with the plasmids carrying the NF-κB binding sequence alone.

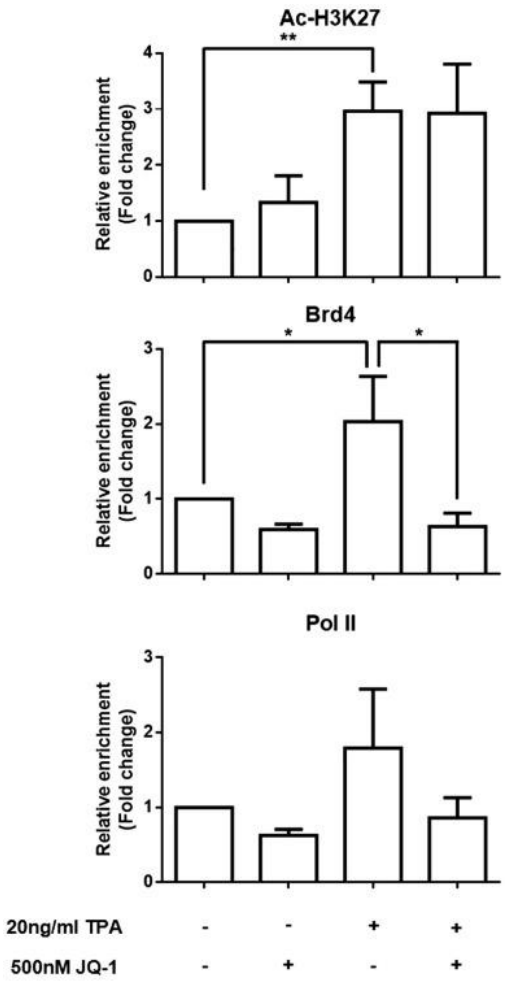

Figure 4.7 Effect of JQ-1 on the recruitment of the transcriptional machinery to COX2 under TPA stimulation. ChIP assays were performed to analyze the enrichment of H3K27Ac, Brd4, and Pol II on the promoter or transcription start site of COX2 in JB6 P+ cells. The immunoprecipitated DNA was used as a template for qPCR and the enrichment was quantified as the proportion ratio of its self- input. Relative fold change was then calculated by normalizing the ratio to that of the vehicle control. *P<0.05 and **P<0.01 indicate significant differences between the TPA-treated group and the indicated groups.

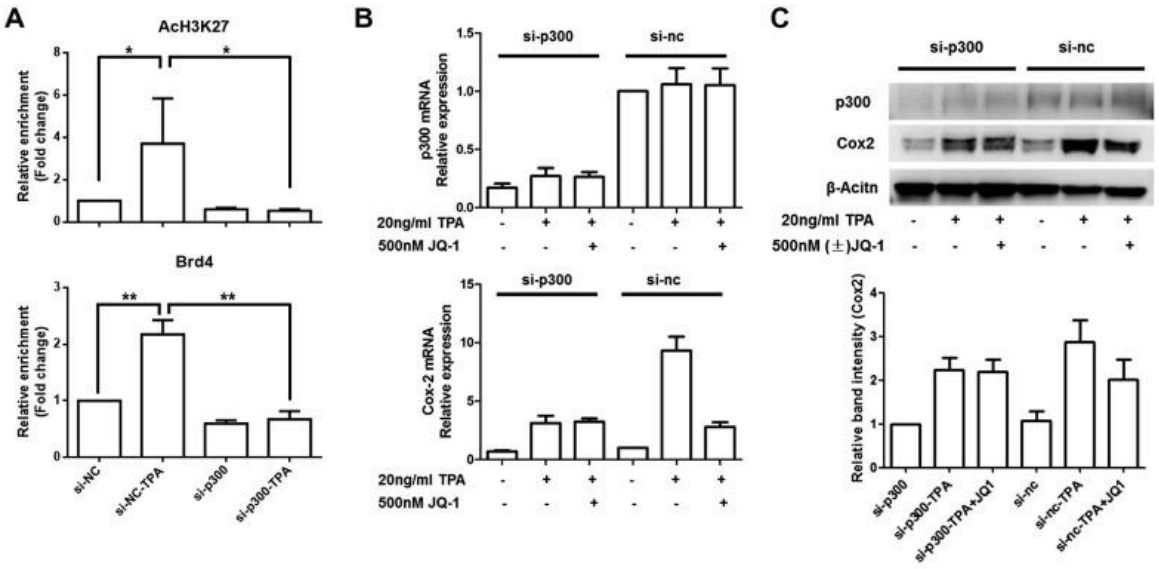

Figure 4.8 JQ-1 inhibits the p300-dependent activation of COX2. JB6 P+ cells were transfected with siRNA against p300 or non-specific negative control, and the cells were then further treated with DMSO, 20 ng/ml TPA alone or 20 ng/ml TPA in combination with 500 nM JQ-1 for 24 h. ChIP assays were performed to examine the enrichment of H3K27Ac and Brd4 after TPA stimulation in the p300 or negative control siRNA treated cells. The mRNA and protein levels of COX2 were assessed respectively. A) TPA increased the Ac-H3K27 enrichment on COX2 gene promoter and Brd4 enrichment on TSS. Knock down of p300 attenuated these TPA induced alterations. B) The mRNA levels of p300 and COX2 were determined by quantitative PCR. C) Representative western blot images of p300 and COX2 protein levels. Transfection of p300 siRNA attenuated TPA-induced COX2 expression at both the mRNA and protein levels. JQ-1 treatment did not show further inhibitory effects after p300 knockdown.

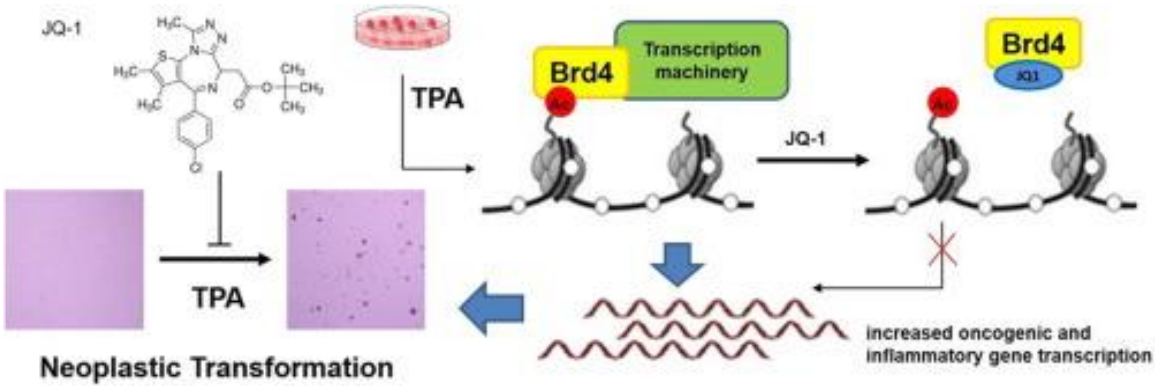

Figure 4.9 Summary of Chapter 4

Chapter 5 Phenethyl isothiocyanate (PEITC) suppresses prostate cancer cell invasion epigenetically through regulating microRNA-194[13,14,15]

5.1 Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies in men in the U.S. Although it is potentially curable when it occurs inside the prostate, PCa bone metastasis causes severe pain and greatly contributes to disease mortality. Tumor metastasis is a complex multistep process that includes the escape of neoplastic cells, integrating intravasation, circulation and target-organ colonization (260). From cellular and molecular aspects, the metastatic potential of a tumor cell depends on its interactions with homeostatic factors that are associated with tumor cell growth, survival, angiogenesis, invasion and metastasis (261). Genetic and epigenetic factors together control gene transcription programming, which is crucial for maintaining tissue homeostasis. In addition to DNA methylation and histone modifications, microRNA (miRNA)-mediated regulation represents another level of epigenetic control of gene expression.

miRNAs are widely found as a class of short, non-coding RNAs of 20-22 nucleotides that post-transcriptionally repress their target gene expression through complementary pairing with the 3'-untranslated regions (UTRs) of cognate mRNAs (262,

[13] Part of this chapter has been published in Mol Nutr Food Res. 2016 Jun;60(6):1427-36.
[14] **Keywords**: phenethyl isothiocyanate, prostate cancer, microRNA, miR-194, cell invasion
[15] **Abbreviations**: BMP1, bone morphogenetic protein 1; CRPC, castration-resistant prostate cancer; miRNA, microRNA; MMP, matrix metalloproteinase; PCa, prostate cancer; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; 3'-UTR, 3'-untranslated region.

263). Altered miRNA levels have been linked to the development and progression of human PCa in clinical observations (264, 265). Functionally, certain miRNAs have been categorized as either tumor suppressors or oncogenes (266). In PCa, the miR-15a–miR-16-1 cluster (267), the Let-7 family (268), miR-34 (269), and miR-200 (270) have been identified as tumor suppressors, whereas miR-21 (271) and miR-125b (272) promote PCa tumorigenesis. miRNAs play an essential role in metastasis, possibly due to their regulatory functions in cell adhesion, neovascularization and tissue invasion networks (273).

Numerous epidemiological and pharmacological studies have suggested that dietary consumption of cruciferous vegetables confers substantial beneficial effects on reducing the risks of cancer in humans, including PCa (274, 275). Therefore, a group of bioactive sulfur-containing compounds named glucosinolates, which are abundant in cruciferous vegetables, have become a great interest (276). Their breakdown products, isothiocyanates (ITCs) such as sulforaphane (SFN) and phenethyl isothiocyanate (PEITC), have been extensively evaluated as chemopreventive agents (81). Studies have shown that PEITC exerts promising anti-cancer effects in PCa cell lines and xenografts *in vitro* and several preclinical PCa animal models *in vivo* (277, 278). More recently, emerging evidence has also shown that PEITC has the potential to modulate epigenetic events, such as DNA methylation (279), histone modifications (280), and miRNAs (281), all of which may be involved in carcinogenesis.

In this study, we examined the effects of PEITC treatment on miRNA expression in PCa cells. Among the most altered miRNAs, we found that miR-194 down-regulates oncogenic matrix metalloproteinase (MMP) 2 and MMP9 expression by targeting bone

morphogenetic protein 1 (BMP1), consequently accounting for the effects of PEITC on suppressing PCa cell metastasis.

5.2 Materials and methods

5.2.1 Cell culture and treatment

PCa LNCaP and PC3 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37℃ in a humidified 5% CO_2 atmosphere. PEITC was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cells were grown to approximately 80% confluence and then treated with the medium containing PEITC at indicated concentrations for 24 h. DMSO at 0.1% was used as the vehicle control.

5.2.2 RNA isolation, miRNA profiling and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the treated cells using miRCURY™ RNA Isolation Kits (Exiqon, Woburn, MA). miRNA profiling was performed by using the mean values from a pre-printed miRCURY LNA microRNA Array (Exiqon, Woburn, MA). miRNA samples were labeled using a miRCURY LNA microRNA Power Labeling Kit, Hy3/Hy5 (Exiqon, Woburn, MA). The hy3/hy5 dyes are spectrally equivalent to the well-known Cy3/Cy5 fluorophores as suggested by the manufacture. A reference miRNA was spiked in each microarray to facilitate comparisons among samples; miRNA samples were labeled with the Hy3, and the reference was labeled with Hy5. After enzyme inactivation, the samples were subjected to microarray hybridization in an HS4000-PRO platform (Tecan,

Mannendorf, Switzerland) at 56 °C for 16 h and then scanned at 535 and 635 nm. Data analysis was performed using GeneSpring software version 7.2 (Agilent Technologies).

To measure individual miRNA levels, reverse transcription was performed with 500 ng total RNA and certain miRNA-specific primers using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). To determine gene mRNA expression levels, first-strand cDNA was synthesized from 500 ng total RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Grand Island, NY) according to the manufacturer's manual. The cDNA was used as the template for qPCR in the ABI7900HT system (Applied Biosystems). The sequences of the primers used for PCR amplifications are listed in Table 5.1.

5.2.3 Transfection of has-miR-194-5p mimic and inhibitor

We utilized a miRCURY LNA microRNA Mimic/Inhibitor (Exiqon, Woburn, MA) of hsa-miR-194-5p to test the cellular functions of miR-194. For transfection experiments, cells were grown to 70% confluence and then treated with a 30 nM has-miR-194-5p mimic/inhibitor using Lipofectamine 3000 reagent (Life Technologies, Grand Island, NY) for 24 h. Negative control miRNA was used in parallel as a control.

5.2.4 Matrigel cell invasion assay

The invasion capacity of PC3 cells was measured using Matrigel-coated Transwell cell culture chambers (8 μm pore size). The cells treated with miR-194 mimic/inhibitor or PEITC, were maintained in serum-free medium for 24 h and were then seeded in the upper chamber of the Transwell insert ($5*10^4$ cells/well). Culture medium containing 10% FBS was placed in the lower chamber. Cells were incubated for 12 h at 37℃ in a humidified 5%

CO_2 atmosphere. The cells penetrated through the Matrigel to the lower surface of the membrane were stained with a Differential Quik Stain Kit (Polysciences, Warrington, PA). Invasive cell numbers were counted under a computerized microscope system with the Nikon ACT-1 program (Version 2.20).

5.2.5 Western blotting

The treated cells from above were harvested using radioimmunoprecipitation assay (RIPA) buffer supplemented with a protein inhibitor cocktail (Sigma, St. Louis, MO). The clear supernatants from whole cell lysates were loaded (20 μg/lane) and separated by 4-15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA). Then, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) followed by blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline-0.1% Tween 20 (TBST) buffer. Primary antibodies against MMP2 and MMP9 (Abcam, Cambridge, MA) and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The blots were visualized by using a SuperSignal enhanced chemiluminiscence (ECL) detection system and recorded using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). Densitometry of the bands was analyzed using ImageJ (Version 1.48d, NIH).

5.2.6 Luciferase reporter activity assay

The 3'-UTR of BMP1 mRNA was reverse transcribed, amplified by PCR, then cloned into the pGL4.15 [luc2P/Hygro] (Promega, Madison, WI) luciferase reporter vector. The recombinant plasmid sequences were validated by direct sequencing (Genewiz, South Plainfield, NJ). For the luciferase activity assay, PC3 or HEK293 cells were seeded into 12-well plates until they reached 80% confluence and were then co-transfected with 30 nM

miR-194-5p mimic/inhibitor and 300 ng of the reporter plasmid using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's manual; 100 ng of the pSV-β-Galactosidase vector was co-transfected as internal control. After 24 h of incubation, the cells were lysed in 1X Reporter Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was measured with the cell lysate using a Sirius luminometer (Berthold Technologies, Pforzheim, Germany). The luciferase activities were normalized with β-Galactosidase activity and were reported as fold changes compared with the negative control miRNA.

5.2.7 RNA interference

RNAi Duplex Oligos (siRNA) targeting human BMP1 were designed and synthesized by Integrated DNA Technologies (Coralville, IA). Transfection of siRNA was conducted using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. PC3 cells were seeded in 6-well plates until they reached 80% confluence and were then transfected with 30 nM BMP1 siRNA. After 24 h of incubation, the cells were collected for RNA and protein extraction. A universal negative control siRNA was similarly prepared in parallel and used as the control. The siRNA sequences are listed in Table 5.2.

5.2.8 Statistical analysis

The data are presented as means ± SD. The statistical analyses were performed using a one-way analysis of variance (ANOVA). Statistically significant differences among the means were set at $*p<0.05$, $**p<0.01$, and $\#p<0.001$.

5.3 Results

5.3.1 PEITC alters miRNA expression in PCa cells

An oligonucleotide microarray was used to analyze the miRNA expression profiles in PCa LNCaP cells following PEITC treatment. A hierarchical cluster analysis was employed to evaluate differential miRNA expression among the samples. The clustering was performed on log2 (Hy3/Hy5) ratios that passed the filtering criteria for variations between each sample versus control. As shown in Fig 1A, samples treated with PEITC and curcumin (another widely investigated chemopreventive agent) clustered together on the right side. The names of top altered miRNAs are also listed in Fig 1A. Because we used a single sample without replications on the microarray, qPCR validation was performed on several top altered miRNAs. In agreement with the microarray results, PEITC treatment decreased miR-106a/b and miR-695 levels in LNCaP cells (Figure 5.1B), whereas miR-194 levels were up-regulated in both the LNCaP and the PC3 cells (Figure 5.1C).

5.3.2 miR-194 suppresses PC3 cell invasiveness *in vitro*

Among the miRNAs that were most frequently altered by PEITC treatment, miR-194 has been reported to be a potential tumor suppressor that is inversely associated with tumor invasion in gastric cancer (282). In this study, we tested whether miR-194 contributes to the effects of PEITC in suppressing prostate cancer cell invasion, by using Matrigel-coated Transwell cell culture chambers. After 12 h of incubation, the cells that migrated from the upper chamber to the lower chamber on the basal side of the membrane were captured (Figuer 5.2A) and counted (Figure 5.2B). Treatment with PEITC (2.5 μM) or the miR-194 mimic reduced the number of cells that penetrated the Matrigel by 55%

and 52%, respectively. In contrast, the miR-194 inhibitor antagonized the inhibitory effect of PEITC on cell invasiveness. The transfection efficiency of miR-194 mimics and miR-194 inhibitors were confirmed in parallel before the cells were seeded in the Transwell chamber (Figure 5.2C).

5.3.3 PEITC down-regulates MMP2 and MMP9 via miR-194

MMPs are enzymes capable of degrading extracellular matrix proteins. Among all MMPs, oncogenic MMP2 (gelatinase-A) and MMP9 (gelatinase-B) are deeply involved in tumor migration, invasion and metastasis for various human cancers (283). We tested whether miR-194 would decrease the levels of MMP2 and MMP9 in prostate cancer cells underlying its invasion suppressive function. Upon PEITC treatment or transfection of miR-194 mimic, we found that both mRNA and protein levels of MMP2 and MMP9 decreased, whereas gene expression increased in the cells upon miR-194 inhibition (Figure 5.3A & B).

5.3.4 BMP1 is a direct target of miR-194

Each miRNA may naturally target a series of mRNAs (284). To further examine the mechanism by which miR-194 regulates MMP2 and MMP9 expression, TargetScan (285) and PicTar (286) were used to discover the mRNA targets of miR-194. Surprisingly, neither MMP2 nor MMP9 was predicted to be a miR-194 target according to these two algorithms. Among the candidate mRNAs, we found that BMP1 may play a role in promoting cell migration and invasion through gene ontology analysis (Fig 5.4A). We then cloned the 3'-UTR of BMP1 into a luciferase reporter vector. Co-transfection of the miR-194 mimic and the luciferase construct reduced luciferase activity, indicating that miR-194 directly targets on the BMP1 3'-UTR. Moreover, corresponding with the elevated miR-

194 levels mentioned above, PEITC treatment decreased the luciferase activity of the same vector (Fig 5.4B). In addition to the reporter assay, we performed qPCR to evaluate BMP1 mRNA levels after miR-194 mimic/inhibitor or PEITC treatment. We found that both miR-194 mimic and PEITC treatments decreased BMP1 expression (Fig 5.4C).

5.3.5 BMP1 inhibition decreases cellular MMP levels

Having demonstrated that BMP1 is a potential target of miR-194, we then tested whether BMP1 was involved in the miR-194-mediated down-regulation of MMP2 and MMP9. We carried out gene silencing by using BMP1 siRNA, and the knock down efficacy was confirmed by qPCR (Fig 5.5A). After transfection, the cellular MMP2 and MMP9 expression levels were measured by qPCR and western blotting. We found that BMP1 knockdown attenuates MMP2 and MMP9 expression in PC3 cells at both the mRNA and protein levels (Fig 5.5B & C). Transfection of a negative control siRNA that has no homology to any known mammalian genes was performed in parallel of BMP1 siRNA transfection.

5.4 Discussion

Numerous epidemiologic studies have shown the protective role of glucosinolates against cancer, including PC (278, 287, 288). PEITC is one of the most widely investigated glucosinolate-derived isothiocyanates from cruciferous vegetables, exerting its chemopreventive effects by modulating multiple relevant processes, such as induction of phase II antioxidative/cytoprotective enzymes, inhibition of a prolonged inflammatory response, and regulation of various cellular signaling pathways, including apoptosis, proliferation, angiogenesis, epithelial-mesenchymal transition (EMT), and cancer stem cell

self-renewal (289). In addition, growing evidence has suggested that epigenetic mechanisms, particularly those mediated by miRNAs, are implicated in the chemopreventive effects of PEITC. Consistently with results from previous reports, in the present study, we found that PEITC can decreases MMP2/9 levels and subsequently inhibits PCa cell invasion. Interestingly, we also found that PEITC treatment significantly induces cellular miR-194 expression. Elevated miR-194 levels may account for the attenuated cell invasiveness, suggesting a new mechanism by which PEITC inhibits PCa cell invasion.

In PCa progression, metastatic castration-resistant prostate cancer (CRPC) no longer responds to conventional androgen deprivation therapy and contributes most of PCa death. Although alternative strategies such as chemotherapy and radiotherapy have been introduced for the treatment of CRPC (290), they do not significantly improve survival (291). Therefore, novel treatments are urgently needed and efforts have been made on seeking the miRNA targets (292, 293). miRNA represents a “fine tuning” level of epigenetic regulation that can modulate a wide diversity of biological processes. As such, miRNAs are sensitive to changes in the cellular environment, such as pathological progression and/or chemical interventions. A previous study by Izzotti et al. has shown that the miRNA networks modulated by PEITC are involved in a variety of functions that play roles in protecting environmental cigarette smoke ECS-induced pulmonary carcinogenesis (294). In this study, we identified the miRNAs that were most affected by PEITC treatment in PCa LNCaP cells; miR-194 and miR-659 were induced by PEITC, whereas miR-17, miR-18a, miR-20a, miR-106a/b, miR-301a were down-regulated. Several of these miRNAs are known to be associated with prostatic carcinogenesis and their expressions

were validated by repeated qPCR. The transcriptional regulation of series of miRNAs is known to occur in a polycistronic manner; thus, alteration of one member in the cluster often coincides with alterations of other members in the cluster (295). The miR-17-92 cluster, also known as oncomiR-1 (oncogenic miRNA), is among the best-characterized miRNA clusters (296); it comprises miR-17, miR-18, miR-19a, miR-20a, miR-19b-1 and miR-92a-1. The miR-17-92 cluster was initially found in the 13q31-q32 amplicon in malignant lymphoma (297), which plays an important role in cell cycle regulation, proliferation, apoptosis and other pivotal processes. miR-17-92 overexpression has been widely found in a variety of malignances, including B-cell lymphoma (298), acute myeloid leukemia (AML) (299), hepatocellular carcinoma (300), and breast (301), colon (302), lung (303), and pancreatic (304) cancers. Previous reports have demonstrated that PEITC decreases miR-17 and miR-20a expression, resulting in decreased Myc expression in a reactive oxygen species (ROS)-dependent manner (305). Correspondingly, our result shows that PEITC decreases the expression of several members of the miR-17-92 cluster, including miR-17, miR-18a, and miR-20a, in PCa cell lines. In addition, elevated miR-106 levels have been detected in prostate tumors with high Gleason scores (306), whereas the oncogenic potential of miR-106 has been identified in human T-cell leukemia (307) and colorectal cancer cells (308). Interestingly, we also found that PEITC decreases miR-106a/b levels in PCa LNCaP cells. In summary, the evidence mentioned above indicates that miRNA regulation is part of a mechanism that contributes to the anticancer effect of PEITC. An unexpected observation is that PEITC decreases levels of miR-26a/b, which have long been considered as anti-oncomiRs (309, 310). However, the reduced miR-26a/b levels do not override the anti-invasive activity of PEITC in our study.

The function of miR-194 in prostate cancer has not been evaluated so far, although it is at the top of the list of our miRNA profiling candidates. Recent studies have shown that miR-194 acts as a tumor suppressor in liver (311), gastric (282), and non-small cell lung cancers (312). It is unsurprising that the expression of tumor suppressive miR-194 can be induced by a chemopreventive agent such as PEITC. Gene ontology analysis was conducted among the predicted targets of miR-194 (286), which indicates its potential ability to regulate genes involved in various aspects of the invasion-metastasis cascade. Therefore, a Matrigel Transwell assay was performed to investigate the relationship between miR-194 expression and the invasive growth of PCa cells. The number of the PC3 cells that invaded through the membrane was reduced by 55% after ectopic expression of miR-194. In contrast, when a miR-194 inhibitor was applied in the presence of PEITC, the invasiveness of the cells was enhanced. In addition, it has been reported that ectopic expression of miR-194 has little effect on cancer cell proliferation *in vitro* (312). Thus, we exclude the possibility that the observed effects on PCa invasion are due to toxicity from ectopically expressing miR-194. This result suggests that miR-194 at least partially contributes to the anti-invasive effect of PEITC.

Numerous potential targets of miR-194 have been identified in a variety of cells and tissues. In multiple myeloma, miR-194 targets MDM2 expression, which in turn enhances the therapeutic activity of MDM2 inhibitors by increasing their p53-activating effects (313). It has also been reported that miR-194 overexpression inhibits the proliferation, migration, and invasion of osteosarcoma cells by targeting CDH2 (N-cadherin) and insulin-like growth factor receptor 1 (IGF1R) (314). Moreover, high levels of miR-194 have been observed in hepatic epithelial cells, which suppress the invasion and

migration of mesenchymal-like cancer cells by down-regulating several genes involved in the EMT (311). Correspondingly, miR-194 represses the expression of the oncogene polycomb complex protein-1 (BMI-1), which accounts for cell invasion and EMT in endometrial cancer cells (315). In this study, we found that miR-194 has direct complementary pairing with the 3'-UTR of BMP1 mRNA. Previous high-throughput screens have identified BMP1 RNA sequences as among the most up-regulated transcripts in human tumor endothelium associated with angiogenesis (316). BMP1 reportedly activates transforming growth factor beta-1 (TGFβ1) signaling via cleavage of latent TGFβ-binding protein (LTBP1) (317); TGFβ activation, along with subsequent roles played by MMPs (318), is important in the tissue remodeling associated with morphogenesis and to cancer metastasis. Thus, BMP1 suppression by miR-194 reduces TGFβ activity, resulting in decreased MMP2 and MMP9 expression, thereby attenuating the invasive capacity of PCa cells. Our present study, together with the aforementioned findings, suggests that miR-194 could modulate multiple steps in the invasion and metastasis cascade.

Like other RNA molecules, miRNAs are transcribed from DNA as immature primary miRNAs (pri-miRNAs) (319) and then are processed by Drosha into precursor miRNAs (pre-miRNAs), which are approximately 70 nt long (320). Finally, the pre-miRNA hairpins are transported to the cytoplasm and cleaved by Dicer to form mature miRNAs (321). Based on this biogenesis diagram of miRNA, it should be noted that the initial transcription of miRNAs can be regulated by transcriptional factors. Recent studies have indicated that hepatocyte nuclear factor 1α (HNF-1a) (322) and p53 (313) can bind to the promoter elements of the gene encoding pri-miR-194, thereby inducing the cellular

levels of miR-194. It is known that PEITC induces p53 protein expression and p53-dependent transactivation (323). Thus, PEITC may potentially stimulate transactivation of the miR-194 gene in a p53-dependent manner. Furthermore, it is also possible that the transcription process of miR-194 could be regulated via epigenetic mechanisms such as DNA methylation and histone modifications. Aberrant DNA methylation associated epigenetic silencing may affect the miRNA network. Promoter hypermethylation of miR-194 has been found in the chorioamniotic membranes of early onset pre-eclampsia (EOPE) sample (324). On histone modification scope, metastasis-associated gene 1 (MTA1) is an integral part of the nucleosome remodeling and histone deacetylation (NuRD) complex that functions as a co-regulator in transcriptional programs (325). Gene knock down of MTA1 in human lung cancer cells alters the microRNA expression profile in human lung cancer cells, including miR-194 (326). On the other hand, putatively miR-194 could target genes involved in epigenetic regulation, such as DNA methyltransferase-3a (DNMT-3a), MeCP2 (293) and EP300 (327), which indicates potential crosstalk of miR-194 on different epigenetic levels. It is of great interest to further explore the precise mechanisms by which miR-194 expression is regulated in PCa.

5.5 Conclusion

In conclusion, we demonstrated that miR-194 up-regulation helps PEITC suppress PCa cell invasion, suggesting a new mechanism by which PEITC modulates PCa metastasis. As shown above, PEITC may affect a wide range of miRNAs, and each miRNA may have multiple mRNA targets; therefore, PEITC may have a broader functional impact on PCa cells at the miRNA level. Investigating these effects of PEITC will be of interest in future studies.

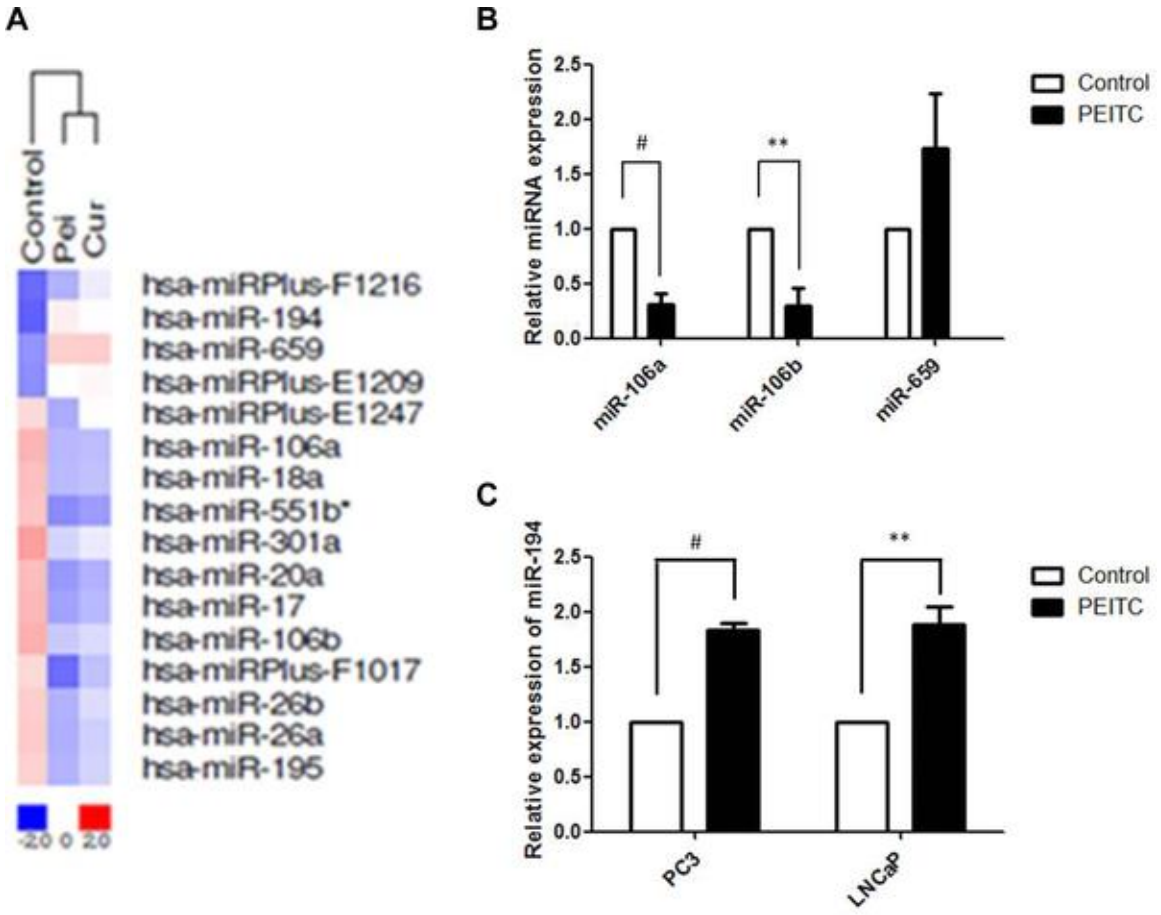

Figure 5.1 PEITC alters the miRNA profile in PCa cells. A) Hierarchical cluster analysis relative to the expression profiles of miRNAs in untreated group and cells treated with either PEITC or curcumin. miRNA expression intensity is represented according to a color scale ranging between blue (low) and red (high), only the most altered miRNAs are shown with their names; B) qPCR validation of miR106a, miR106b, miR-659 levels in LNCaP cells treated by PEITC; C) PEITC induces miR-194 levels in both LNCaP and PC3 cells.

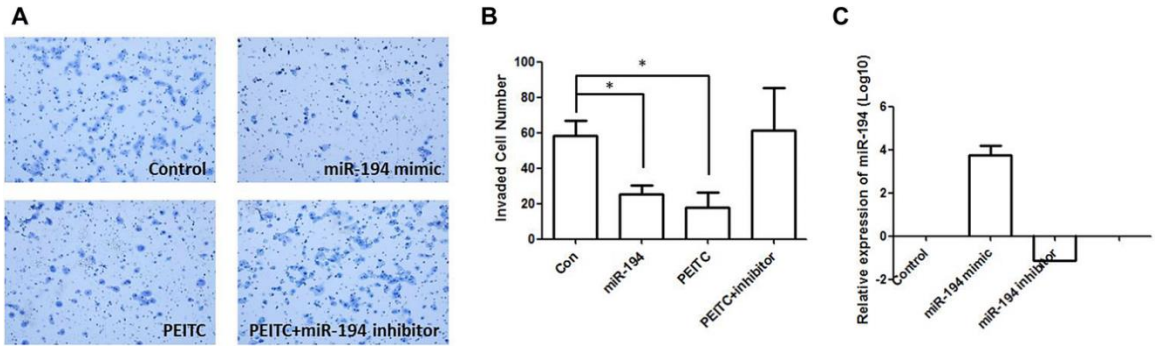

Figure 5.2 miR-194 suppresses PC3 cells invasiveness. Matrigel Transwell cell invasion assay was performed to test the effect of miR-194 on the invasive ability of PC3 cells. PC3 cells were treated with miR-194 mimic, 2.5μM PEITC, and a combination of miR-194 inhibitor and 2.5μM PEITC prior to seeding in the chamber. The cells that invaded the basal side of the membrane were captured under microscopy and the cell numbers were counted. A) representative imagines of the Transwell assays for cell migration; B) invasion ability of PC cells quantified by counting the number of cells that invaded the underside of the membrane; C) validation of transfection efficiency of miR-194 mimic and miR-194 inhibitor by qPCR. Data are presented as the mean ± SD from at least three independent experiments.

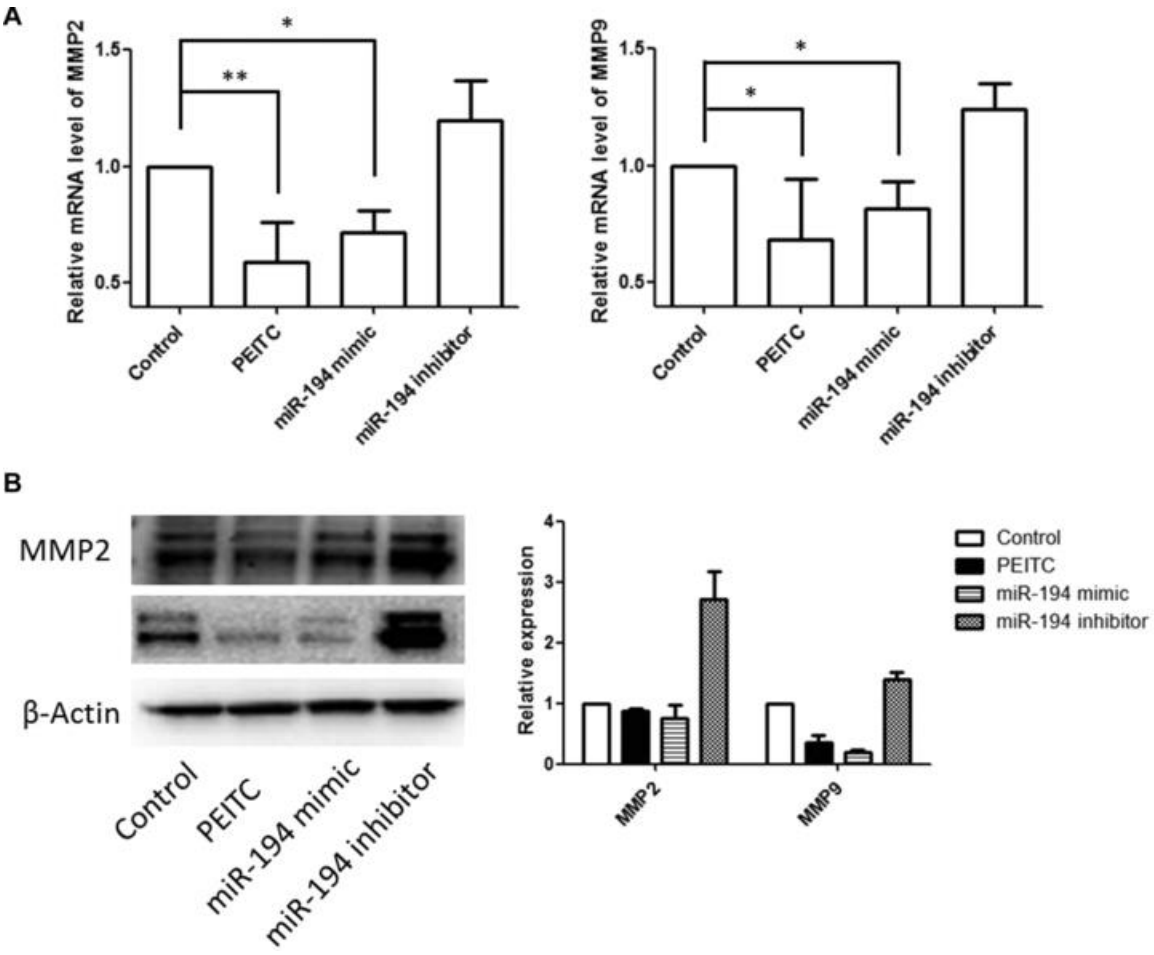

Figure 5.3 miR-194 decreases the expression of MMP2 and MMP9 in PCa cells. The mRNA and protein levels of MMP2 and MMP9 were determined using qPCR and Western blotting following the indicated treatments, respectively. A) the relative mRNA levels of MMP2 and MMP9; B) representative immunoblotting bands of MMP2 and MMP9 protein levels. The relative abundance of each target protein was calculated by normalizing the band intensity to β-actin using ImageJ. The bar chart in the right panel presents the mean ± SEM of three independent experiments.

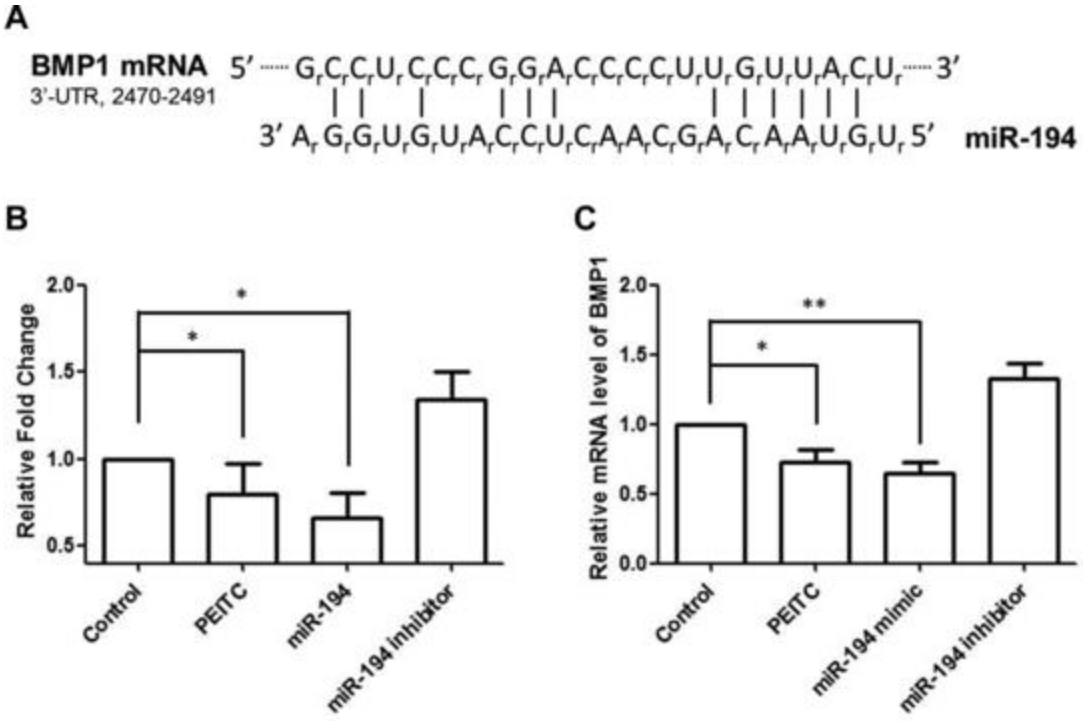

Figure 5.4 BMP1 is a direct target of miR-194. The BMP1 3'-UTR was amplified from cDNA library and inserted into pGL4.15 vector. The luciferase reporter assay was performed using the recombined construct with PEITC, miR-194 mimic and miR-194 inhibitor treatment. A) sequence of miR-194 and the predicted complementary pairing between miR-194 and human BMP1 3'-UTR (2470-2491); B) relative fold change of luciferase activities tested on the BMP1 3'-UTR reporter genes; C) relative BMP1 mRNA levels after treatment of PETC, miR-194 mimic, and miR-194 inhibitor.

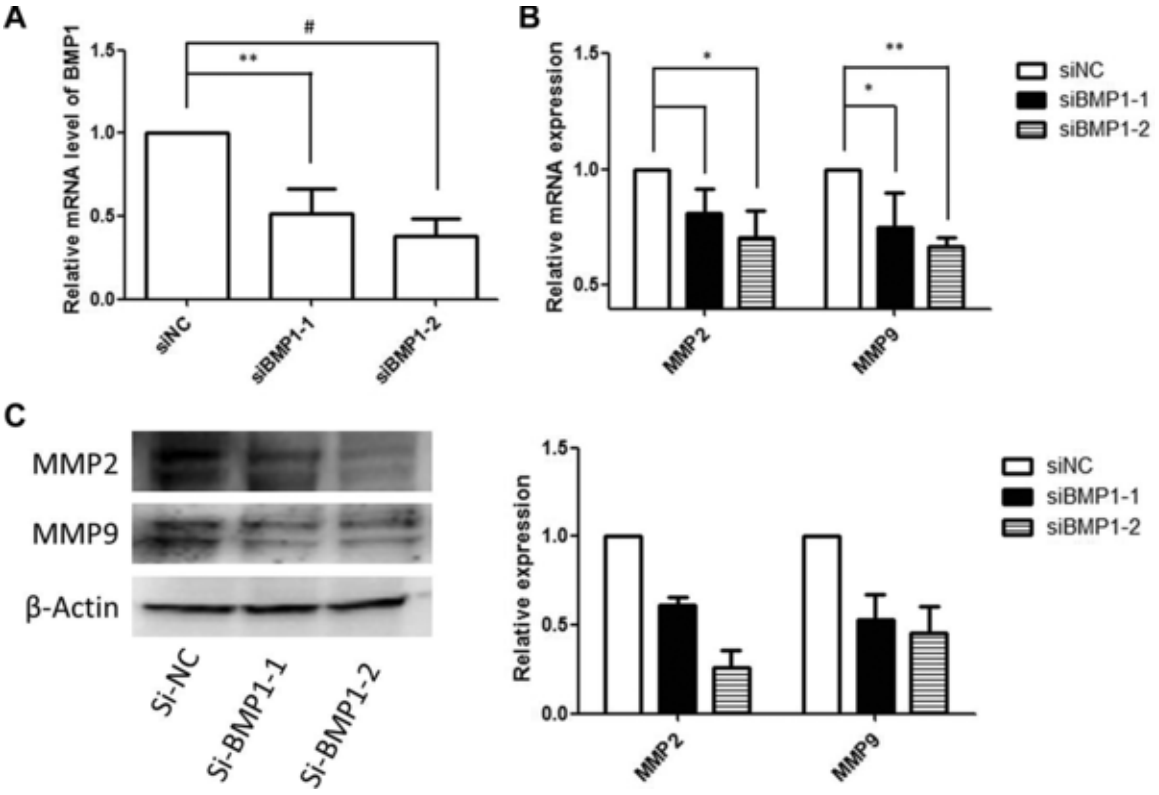

Figure 5.5 Downregulation of BMP1 mediates the decreased expression of MMP2 and MMP9. After transfection of BMP1 siRNA, the mRNA and protein levels of MMP2 and MMP9 were determined using qPCR and Western blotting. (A) validation of BMP1 siRNA silencing efficiency by qPCR; B) the relative mRNA levels of MMP2 and MMP9; (B) representative immunoblotting bands of MMP2 and MMP9 protein levels. The relative abundance of each target protein was calculated by normalizing the band intensity to β-actin using ImageJ. The bar chart in the right panel presents the mean ± SEM of three independent experiments.

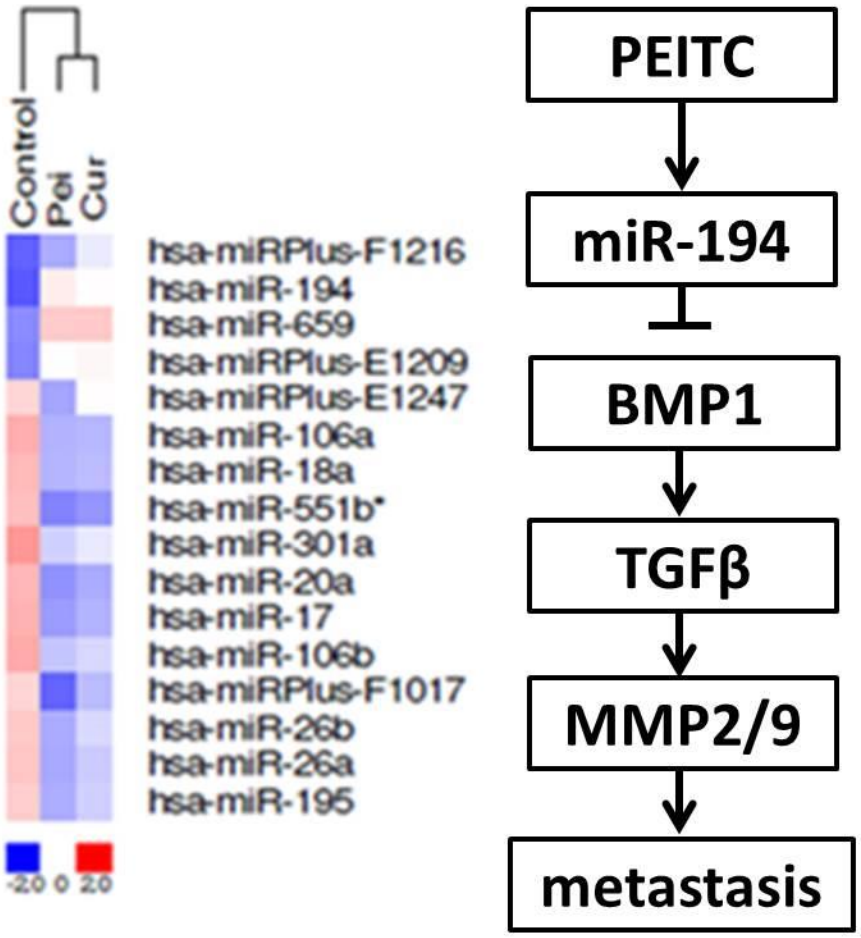

Figure 5.6 Summary of chapter 5

Table 5.1 List of qPCR primers

Primer name	Sequence (5'-3')
MMP2 sense	TAC AGG ATC ATT GGC TAC ACA CC
MMP2 anti-sense	GGT CAC ATC GCT CCA GAC T
MMP9 sense	TGT ACC GCT ATG GTT ACA CTC G
MMP9 anti-sense	GGC AGG GAC AGT TGC TTC T
BMP1 sense	CTC CCC TGA ATA CCC CAA TG
BMP1 anti-sense	ACC TCC ACA TAG TCG TAC CAG
GAPDH sense	ACA TCG CTC AGA CAC CAT G
GAPDH anti-sense	TGT AGT TGA GGT CAA TGA AGG G

Table 5.2 siRNA sequence

siRNA Target		Sequence (5'-3')
BMP1 siRNA-1	Sense Strand:	rCrUrCrCrCrUrGrArGrCrCrUrArUrCrGrUrCrUrCrCrArC
	Antisense Strand:	rCrArGrUrGrGrArGrArCrGrArUrArGrGrCrUrCrArGrGrGr ArGrUrU
BMP1 siRNA-2	Sense Strand:	rGrGrUrArCrArGrCrArGrGrCrUrGrUrGrGrArUrCrUrCrA
	Antisense Strand:	rUrCrUrGrArGrArUrCrCrArCrArGrCrCrUrGrCrUrGrUrAr CrCrUrG

Appendix 1 MicroRNAs: new players in cancer prevention targeting Nrf2, oxidative stress and inflammatory pathways[16,17,18]

A1.1 Introduction

Cancer chemoprevention involves the use of chemical agents that naturally occur in food or are administered as pharmaceuticals to inhibit or reverse the carcinogenic process. Increasing evidence indicates that epigenetic modifications can initiate cancer. Unlike genetic mutations, epigenetic changes are modifications of gene expression that occur in the absence of alterations in DNA sequences. Thus, epigenetic changes have been identified as new targets for chemopreventive strategies because they are considered to be reversible via certain chemicals. In addition to DNA methylation and histone modifications, gene expression is regulated by microRNAs (miRNAs) in what is considered to be a "fine-tuning" mechanism of epigenetic modulation. MiRNAs are endogenous small non-coding RNAs of 20-22 nucleotides that repress gene expression through the degradation of mRNA or the inhibition of protein translation (262, 263). Diverse miRNAs have been identified to control cell growth, the cell cycle, cell differentiation, tumor suppression and apoptosis (328-330). Genome-wide profiling has shown that the miRNA expression patterns of healthy and pathological tissues vary, as do those of different types of cancer. Therefore, increasing efforts have focused on analyzing these miRNA expression pattern alterations to identify novel cancer biomarkers and therapeutic targets. Interestingly, as shown in

[16] Part of this chapter has been published in Curr Pharmacol Rep. 2015 Feb;1(1):21-30.
[17] **Keywords**: microRNA; carcinogenesis; inflammation; nrf2; redox homeostasis
[18] **Abbreviations**: RISC, RNA-induced silencing complex; GSH, glutathione; Keap1, Kelch-like ECH-associated protein 1; AP-1, activator protein-1; SOCS, suppressor of cytokine signaling.

Table 1, dietary chemopreventive agents from widely different sources have also been evaluated as modulators of miRNA expression in a variety of cancers (331).

Moreover, a number of naturally occurring chemopreventive compounds possess anti-oxidative and anti-inflammatory properties that are associated with their protective effects against tumorigenesis. Oxidative stress can be a consequence of the increased generation of reactive oxygen/nitrogen species (ROS/RNS) and/or decreased functioning of the anti-oxidative stress defense systems of the body (332). In immune cells, endogenous ROS/RNS are generated to eliminate invading pathogens (333, 334). In the context of oxidative stress, the secretion of a large amount of ROS/RNS recruits more activated inflammatory immune cells. When the crosstalk between inflammation and oxidative stress becomes chronic, excessive cellular ROS/RNS is produced (335-337). This exacerbating loop can result in the oxidation of intracellular proteins, lipids and nucleic acids, leading to aberrant genetic changes and/or epigenetic alterations such as the dysregulation of oncogene and tumor-suppressor gene expression (221, 337, 338).

Phase II detoxifying/antioxidant enzymes, such as glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), heme oxygenase-1 (HO-1), NADP(H):quinone oxidoreductase (NQO), glutamate cysteine ligase (GCL) and gamma glutamylcysteine synthetase (γGCS), among others, produce a cytoprotective environment by reducing the toxicity of reactive intermediates, which augments cellular defense against stress and maintains intracellular redox homeostasis (335, 339). The genes that encode detoxifying/antioxidant enzymes typically share a consensus cis-element in their promoter regions that is known as the antioxidant response element (ARE) or electrophile response element (EpRE). The transcriptional activation of ARE-mediated genes is primarily

regulated by nuclear factor erythroid-2 related factor-2 (Nrf2 or NFE2L2), a helix-loop-helix basic leucine zipper transcription factor. Extensive research both in vitro and in vivo has demonstrated the important role of Nrf2 as a molecular target for naturally derived chemopreventive agents.

How miRNAs interact with anti-oxidative and anti-inflammatory pathways in diverse physiological and pathophysiological processes remains to be fully elucidated. Given that the Nrf2/ARE and inflammatory pathways have long been the focus of cancer prevention studies, uncovering the relationship between miRNAs and these signaling pathways could reveal an important regulatory mechanism that blocks carcinogenesis.

A1.2 MiRNAs and carcinogenesis

The relationship between aberrant miRNA expression and carcinogenesis may be attributed to the fact that greater than 50% of the genes that encode these miRNAs are located in the cancer-associated regions of fragile sites; these sites exhibit chromosomal instability via events associated with amplification, translocation or deletion (340). Based on the properties of their target genes, miRNAs can function as either oncogenic miRNAs or tumor-suppressive miRNAs. Because miRNAs are commonly expressed as polycistronic transcripts, the dysregulation of one member of the cluster often coincides with the dysregulation of other members of the cluster. Therefore, miRNome-wide aberrations are more likely to be involved in carcinogenesis than mutations in a single miRNA gene that targets one oncogene or tumor suppressor.

A study conducted by Calin et al. was the first to establish the existence of altered levels of the miR-15a/miR-16-1 cluster in B-cell chronic lymphocytic leukemia (CLL)

(341). Both miRNAs are located at chromosomal position 13q14.3, which is a region that is often deleted in CLL and prostate cancer (342, 343). One of the identified targets of miR-16-1 is B-cell lymophoma 2 (BCL2), which is an anti-apoptotic gene associated with cell survival and carcinogenesis. Therefore, the impaired expression of miR-15a/miR-16-1 is inversely correlated with BCL2 expression in CLL; additional genes affected by this cluster include MCL1, ETS1, and JUN, among others (344). The tumor-suppressive function of this miRNA cluster was further validated by the finding that the overexpression of the miR-15a/16-1 cluster induces apoptosis in the chronic myeloid leukemia MEG-01 cell line by activating the intrinsic apoptosis pathway and inhibiting tumor growth in a nude mouse engraftment model (344, 345). In addition to miR-15 and miR-16, other miRNAs with a tumor-suppressive role (anti-oncomirs) are frequently down-regulated in cancer cells. For example, p53 suppresses the proto-oncogene c-Myc via the induction of miR-146 (346), which is commonly down-regulated in lung and breast cancer and is deleted in prostate cancer (347). However, remarkably, some miRNAs can function as either oncogenic or tumor-suppressive factors depending on the cell type and the pattern of gene expression (348). Most oncomirs down-regulate the expression of tumor suppressors and are overexpressed in cancer cells. For example, the consistent up-regulation of oncogenic miR-21 expression has been reported in various cancers, including those of the breast, colon, lung, stomach, and pancreas, as well as squamous cell cancer (349). Taken together, these data indicate that profiling differential miRNA patterns in a variety of cancer types may provide potential cancer-specific “miRNA signatures” for diagnostic and therapeutic applications.

Although a number of studies have described the alteration of miRNAs in cancer cells in response to various chemopreventive agents (350), the mechanisms underlying the changes in the expression and the anticancer activities of miRNA induced by these phytochemicals remain to be fully elucidated. Recent investigations into the causes and consequences of miRNA dysfunction indicate that miRNAs are subject to higher levels of control regulating both miRNA biogenesis and function. For example, environmental factors, such as redox homeostasis and inflammatory conditions, play an important role in the modulation of miRNAs.

A1.3 Redox homeostasis regulates miRNA biogenesis

To date, few reports have indicated that Nrf2 regulates the transcriptional activities of the genes encoding miRNAs through Nrf2/ARE-mediated mechanisms. However, several clues in this rapidly developing field support the hypothesis that miRNA biogenesis can be affected by cellular redox homeostasis, which is maintained by the Nrf2 pathway under a number of physiological conditions. The biogenesis of miRNAs occurs similarly to that of other RNA molecules, which begins with DNA transcription. Immature primary miRNAs (pri-miRNAs) are synthesized via the transcription of the miRNA genes by RNA polymerase II (319). The pri-miRNAs are then processed by a nuclear microprocessor complex consisting of RNase III (Drosha) and the co-factor DiGeorge critical region 8 (DGCR8) to form precursor miRNAs (pre-miRNAs) that are approximately 70nt long (320). After their production in the nucleus, the pre-miRNAs are transported to the cytoplasm by the nuclear export factor exportin 5 (XPO5) where they are bound by the RNase III-type endonuclease, Dicer, which can cleave the hairpins of the pre-miRNAs to form double-stranded RNAs (321). Finally, the mature miRNAs are assembled into a

multi-protein effector RNA-induced silencing complex (RISC) with members of the Argonaute protein family (Ago 1-4), which are the essential core components of the RISC (351). Recently, increasing evidence has shown that the key molecules in each step of miRNA biogenesis are affected by the cellular redox environment. For instance, the binding of ferric heme enhances the activity of DGCR8 (352, 353), which plays a role in the maturation of all of the canonical miRNAs. Therefore, the Nrf2-regulated antioxidant enzyme HO-1 affects miRNA biogenesis by competing for the heme substrate, thereby decreasing the activity of DGCR8. This finding is supported by the observation that the overexpression of HO-1 inhibits the differentiation of murine myoblasts by down-regulating miRNA biogenesis, thus altering gene expression by modulating the global level of functional miRNAs (354). In addition, the transporter activity of XPO5 can be affected by oxidative stress in a manner similar to that in other nuclear exporters (355), resulting in another molecular hub in which the cellular redox conditions may affect the miRNA transcriptome. The expression of cytoplasmic Dicer has been shown to be down-regulated by aging and hydrogen peroxide-induced oxidative stress in rat cerebral microvascular endothelial cells (CMVECs) (356). Aging-associated vascular dysfunction in rats is characterized by decreased Nrf2-mediated antioxidant signaling and increased oxidative stress (357). Additionally, Dicer protein levels have also been found to be down-regulated in aged human adipocytes, 3T3-F442 preadipocytes, and JAR trophoblast cells exposed to hydrogen peroxide (358, 359). These findings further support the hypothesis that miRNA biogenesis is affected by redox dysregulation via Dicer. Interestingly, through the in silico screening of transcription factor binding sites and the comparative database analysis of DNA sequences, ARE consensus sequence was discovered in the promoter region of

human and mouse Dicer genes. The existence of this consensus sequence may partially explain why chemopreventive phytochemicals, such as sulforaphane and resveratrol, restore the expression of Dicer in CMVEC cells (356). In addition to the key molecules mentioned above, two families of enzymes, the DNA methyltransferase (DNMT) and histone deacetylase (HDAC) families, can regulate the expression of genes encoding miRNAs via epigenetic mechanisms (360). Both DNMT and HDAC activities are affected by oxidative stress (52), providing other routes by which cellular redox signaling may alter miRNA biogenesis. Furthermore, miRNAs themselves can be modified by ROS, resulting in the alteration of their integrity, stability, binding affinity, and function, thereby contributing to perturbed redox signaling-associated disease mechanisms (361). Because the knockout of the Nrf2 gene significantly impairs cellular redox homeostasis, it is feasible that miRNA processing is altered by cellular Nrf2 levels, thereby resulting in changes of the expression of the miRNAs that modulate antioxidant responses and/or exacerbate oxidative stress.

A1.4 Role of miRNAs in regulating the Nrf2 pathway

One interesting mode of miRNA action is the ability to self-regulate. As mentioned above, miRNAs can participate in the regulation of their own biogenesis by altering redox homeostasis. miRNAs have been observed to be involved in the posttranscriptional regulation of Nrf2 levels. Using an in-silico analysis, Narasimhan et al. demonstrated that the ectopic expression of four miRNA mimics, miR-27a, miR-142-5p, miR-144, and miR-153, can affect the nucleo-cytoplasmic level of Nrf2 protein in a Keap1-independent manner (362). This observation together with the decreased abundance of Nrf2 mRNA by these miRNA mimics indicates that Nrf2 is a direct target of these miRNAs. Thus,

inefficient activating ability of Nrf2 results in diminished GCLC and GSR expression, leading to a decrease in cellular glutathione (GSH) levels. miR-144 was also found to be highly expressed in reticulocytes from patients with sickle cell disease, which may attenuate the antioxidant defense capacity by altering Nrf2 signaling (363). In human mammary epithelial cells and the breast cancer MCF-7 cell line, miR-28 affects Nrf2 mRNA stability by facilitating degradation, which may also modulate the rate of tumor cell growth (364). A systematic analysis of the Nrf2 interactome and regulome by Papp et al. revealed that 85 miRNAs are predicted to bind Nrf2 mRNA to down-regulate its translation (365). Among these, 63 miRNAs were defined as potential members of negative feedback loops for Nrf2 signaling in which an activating transcription factor could increase the level of expression of miRNAs that down-regulate Nrf2. Additionally, positive feedback loops exist whereby an activated transcription factor represses the expression of Nrf2-downregulating miRNAs to enhance Nrf2 translation.

Given that the cellular activity of Nrf2 can be regulated by factors other than miRNAs, such as Kelch-like ECH-associated protein 1 (Keap1), small masculoaponeurotic fibrosarcoma (Maf), BTB and CNC homolog 1 (Bach1), and Parkinson protein 7 (PARK7/DJ-1), the miRNAs that interact with these Nrf2 modulators would also be expected to regulate Nrf2/ARE-mediated signaling in an indirect manner. Keap1 serves as a suppressor of the Nrf2 pathway by binding to Nrf2 and facilitating its ubiquitination and degradation. In the human breast cancer cell line MDA-MB-231, miR-200a can target Keap1 mRNA, leading to its degradation (366). In the same study, Eades et al. found that the epigenetic silencing of miR-200a may contribute to the dysregulation of Nrf2 activity in breast cancer, whereas restoring miR-200a expression leads to enhanced Nrf2 nuclear

translocation and the activation of NAD(P)H-quinone oxidoreductase 1(NQO1) gene transcription, which consequently inhibits the anchorage-independent growth of breast cancer cells. The transcriptional repressor Bach1, which functions in association with small Maf proteins, sequesters ARE-like enhancers within cells and antagonizes Nrf2 binding until it is inactivated by pro-oxidants (367). Several studies have shown that the miRNAs let-7b, let-7c, miR-98, and miR-196 can down-regulate Bach1 expression in human hepatoma Huh-7 cells (368), thereby increasing Nrf2-mediated HO-1 gene expression and attenuating oxidative stress. Notably, pro-inflammatory miR-155 has also been reported to enhance the induction of HO-1 expression via the inhibition of Bach1 translation (369), which indicates that miRNA regulation could be a potential factor in the cross-talk between the inflammatory and oxidative stress pathways. However, several phytochemicals, such as quercetin, allyl-isothiocyanante and sulforaphane, have been reported to decrease miR-155 levels in lipopolysaccharide-stimulated murine RAW264.7 macrophages; the induction of HO-1 expression was also observed (370, 371). Because interactions between the inflammatory response and Nrf2 pathways may occur at multiple levels, the precise mechanisms by which miR-155 affects the cellular anti-oxidative system remain to be fully elucidated. In addition to directly targeting Nrf2 mRNA, miR-34a has also been reported to regulate sirtuin1 (Sirt1) (372), a nicotinamide adenine dinucleotide-dependent class III HDAC that serves as a crucial regulator of metabolism and aging (373). Following the release of Nrf2 from Keap1, the acetylation of Nrf2 by CREB-binding protein (CBP) has been shown to increase the nuclear localization of Nrf2, thus activating Nrf2-dependent transcription through target gene promoters and enhanced ARE binding, whereas the

deacetylation of Nrf2 by Sirt1 disengages Nrf2 from the ARE region of the downstream gene promoter, thereby resulting in Nrf2 transcription and its subsequent nuclear export.

A1.5 Inflammatory conditions and miRNAs

In addition to inducing the expression of detoxifying and antioxidant enzymes, the Nrf2 pathway appears to mediate a strong anti-inflammatory response (374). This finding is supported by the attenuation of the anti-inflammatory effect of sulforaphane in LPS-treated primary peritoneal macrophages from Nrf2 (-/-) mice (86). Furthermore, Nrf2-KO mice are more susceptible to dextran sulfate (DSS)-induced colitis and to AOM-DSS-induced colorectal cancer (90, 375), which are common models for characterizing chronic inflammation and carcinogenesis. Targeting anti-inflammation is another important approach for cancer prevention. In addition to the presence of oxidative stress, the tumor microenvironment contains various infiltrating immune cells due to to inflammatory stimuli (121, 376). Epidemiological studies suggest that chronic inflammation may contribute to the development of 25% of all cancer cases (377). Through cross-talk with cancer cells and tumor stromal cells, tumor-associated macrophages play key roles in cancer-related inflammation by secreting a number of growth factors, angiogenic factors, proteinases, chemokines, and cytokines (378-380). This inflammatory environment stimulates cell proliferation, cell survival, angiogenesis, cell migration, and tumor metastasis, thereby driving cancer progression.

A variety of miRNAs have been associated with cancer-related inflammation. Of these, miR-21, miR-125b, miR-155, miR-196 and miR-210 are the most extensively studied (381, 382). As mentioned in the previous section, miR-21 is overexpressed in nearly all carcinoma and malignancy types (383), and its expression can also be induced in

human peripheral blood mononuclear cells via LPS treatment (384). Surprisingly, miR-21 is implicated in both positive and negative feedback loops depending on the type of pro-/anti-inflammatory cytokines regulated by miR-21. Similar to miR-21, the level of miR-125b was drastically changed in mouse macrophage RAW264.7 cells challenged with LPS (385) where it functions in preventing excessive pro-inflammatory responses. miR-125b expression is down-regulated in metastatic cutaneous malignant melanomas (386). The abnormal overexpression of miR-155 is often associated with enhanced cytokine production. Increased miR-155 expression in tumors does not appear to result from any gene mutation, which suggests that it could instead be a result of inflammatory/oncogenic regulation (387, 388). MiR-210 has been shown to directly target NF-κB transcripts, which serve as a very important negative feedback regulator of the production of pro-inflammatory cytokines (389).

The expression of miR-155, miR-21, miR-125b, miR-196, and miR-210 is controlled by a number of inflammatory signals originating from cells that participate in innate or adaptive inflammatory responses. The activation of Toll-like receptors (TLRs) and the production of cytokines are the most prominent mechanisms linking the functions of these miRNAs with inflammatory events (390-392). For instance, the transcription levels of both pri-miR-21 and pri-miR-125b were up-regulated by LPS treatment in human biliary epithelial cells through NF-κB activation (393). Furthermore, high levels of miR-155 in diffuse large B-cell lymphoma (DLBCL) are related to the endogenous production of TNF, which induces miR-155 expression in these cells, in turn blocking the expression of SHIP1, promoting the conversion of PIP2 to PIP3 by PI3K and thus further augmenting the pro-inflammatory effects of TNF (394). Additionally, the LPS-induced expression of

miR-155 in macrophages can be inhibited by IL-10, a potent anti-inflammatory cytokine that dampens the expression of pro-inflammatory genes (395). The inhibition of miR-155 expression in the presence of IL-10 is associated with B cell integration cluster gene (BIC) transcription repression via its Ets1 site in a STAT3-dependent manner, and the IL-10-dependent inhibition of miR-155 expression may be a potential mechanism used to halt the immune response after the pathogens have been cleared.

A1.6 MiRNAs link inflammation and cancer

The activation of NF-κB and the activation of the transcription factors of activator protein-1 (AP-1) and the members of the STAT families through nuclear translocation are essential for a balanced immune response under inflammatory conditions. However, their continuous activation or overexpression has been found in many different malignancies (54, 377). MiR-21 and miR-155 expression have been reported to be under the control of NF-κB, AP-1, STATs, and SMADs; thus, the enhanced levels of these miRNAs (which are at the junctions between inflammatory and oncogenic signaling pathways) could at least partially explain the link between inflammation and cancer at the molecular level.

The aberrant activation of the JAK/STAT pathway has been demonstrated to be one of the most important inflammatory signals in malignant cells (396). The level of both miR-21 and miR-155 are affected by the members of the STAT families. For example, the NF-κB-dependent recruitment of STAT3 to the miR-21 promoter leads to the up-regulation of miR-2 expression, which may contribute to both the therapeutic resistance and metastasis of breast cancer cells (397). Stimulating breast cancer cells using the inflammatory cytokines IFN-gamma, interleukin-6 (IL-6) and LPS can significantly up-regulate miR-155 expression (398). In the same study, Jiang et al. found that the suppressor

of cytokine signaling 1 (SOCS1), a negative regulator of JAK/STAT signaling, is an evolutionarily conserved target of miR-155 in breast cancer cells. The overexpression of miR-155 leads to the constitutive activation of STAT3, which promotes the proliferation and transformation of breast cancer cells and breast tumor growth in nude mice (398).

TGF-β is a pleiotropic cytokine that exerts important effects on processes such as fibrosis, angiogenesis, and immunosuppression (399). Typically, TGF-β controls gene expression through transcription factors known as SMAD proteins. However, TGF-β facilitates metastasis by altering the homeostasis of TGF-β signaling in advanced malignancies. Using a bioinformatics approach, SMAD2 was predicted to be a target of miR-155 (400). The overexpression of miR-155 altered cellular responses to TGF-β, consequently modulating tissue repair and remodeling that occur in response to macrophages (400). Additionally, miR-155 directly targets the bone morphogenetic protein (BMP)-responsive transcriptional factor SMAD5 in DLBCLs, resulting in resistance to the growth inhibitory effects of both TGF-β and BMPs via the defective induction of p21 expression and impaired cell cycle arrest (401). MiR-21 is also a downstream effector of TGF-β in facilitating cellular invasion and metastasis. Colon carcinoma organoids have been reported to undergo the endothelial-mesenchymal transition in response to TGF-β; this process can be further accelerated by TNF-α, whereas the level of miR-21 is prominently elevated by the synergistic activities of TGF-β/TNF-α.(402).

The constitutive activation of phosphatidylinositol 3-kinase (PI3K)/AKT signaling is one of the most common molecular signatures of human cancers (403). Under physiological conditions, the phosphorylation of AKT is antagonized and balanced by phosphatases such as PTEN, SHIP1 and phosphatase protein phosphatase 2A catalytic

subunit alpha (PPP2CA). Among these, SHIP1 and PPP2CA have been identified to be targets of miR-155 (394, 404); therefore, a high miR-155 level decreases the level of SHIP1 and PPP2CA expression, thereby resulting in the prolonged activation of the PI3K/AKT pathway. Additionally, a proteomics investigation of diffuse large B-cell lymphoma (DLBCL) revealed that PIK3R1 (p85a), which functions as a cellular suppressor of the PI3K/AKT pathway, is also targeted by miR-155 (405). Furthermore, the lack of PTEN expression is found to be associated with an increased level of miR-21 in numerous malignancies. The inhibition of miR-21 expression in cultured human hepatocellular cancer cells increased the expression of the PTEN tumor suppressor and reduced cell proliferation, migration, and invasion (406).

A1.7 Conclusion and perspective

The deregulation of miRNA expression is implicated in carcinogenesis due to its promotion of cell proliferation, survival, invasion, and metastasis as well as its ability to inhibit cell apoptosis and alter the transcription levels of a variety of genes. Naturally occurring chemopreventive compounds have been reported to dramatically affect miRNA expression to produce anti-cancer effects. However, most of the studies of the effects of dietary phytochemicals on miRNA regulation are highly descriptive, and this field remains in its infancy. Because miRNAs regulate mRNA levels in a “fine tuning” manner, they are sensitive to changes in the cellular environment such as those induced by oxidative stress and inflammatory conditions. Given that a number of dietary chemopreventive agents possess anti-oxidative and anti-inflammatory properties, investigations into the biogenesis, metabolism and function of miRNAs under cellular homeostasis/stress conditions would provide new mechanistic insights into the roles of miRNAs in cancer pathogenesis as well

as into approaches using chemopreventive agents. Finally, current studies investigating the expression of a limited number of miRNAs using antagomir or miRNA-mimicking techniques need to be elaborated upon by further studies that profile the genome-wide miRNome.

Table A1.1 miRNAs regulated by chemo-preventive agents

Compound	*Experimental model*	*miRNA*	*Comments*	*Ref*
Butyrate	HCT-116 cells	miR-106b↓	Induce p21 expression	(407)
Curcumin	MCF7 cells	miR-15a↑, miR-16↑	induce apoptosis	(408)
	A549/DDP cells	miR-136↓, miR-186*↓	induce apoptosis	(409)
	BxPC-3 cells	miR-22↑, miR-199a*↓	anti-tumorigenesis	(410)
	Gemcitabine-resistant MIAPaCa cells and BxPC-3 cells	miR-200↑, miR-21↓	curcumin and analogue CDF	(411)
EGCG	HepG2 cells	miR-16↑, let-7 family↑, miR-20a↑, miR-221↑, miR-125b↓	anti-proliferation	(412)
	PCa xenograft	miR-330↑, miR-21↓	inhibit tumor growth	(413)
Ellagitannin	HepG2 cells	let-7e↑, miR-370↑, miR-373*↑, and miR-526b↑		(414)
Genistein	PC3 cells	miR-221↓, miR-222↓	up-regulation of ARHI	(415)
	PC3 cells	miR-1296↑	repression of MCM2	(416)
	C918 cells (human uveal melanoma)	miR-27a↓	up-regulation of ZBTB10	(417)
	Colo357 and Panc-1 cells	miR-146a↑	inhibition of pancreatic cancer cell invasion	(418)
I3C/DIM	Vinyl carbamate-induced mouse lung tumors	miR-21↓, mir-31↓, miR-130a↓, miR-146b↓, miR-377↓		(419)
	Rats exposed to cigarette smoke	let-7c↑	I3C alone and combine with PEITC	(420)
	MiaPaCa-2, Panc-1, and Aspc-1 cells	miR-200↑	reversal of EMT	(421)

	Colo357 and Panc-1 cells	miR-146a↑	inhibition of pancreatic cancer cell invasion	(418)
PEITC	Rats exposed to cigarette smoke, lung tissues	let-7a↑, let-7c↑, miR-99b↑, miR-123↑, miR-146↑, miR-192↑, miR-222↑,		(420)
	Mice exposed to cigarette smoke, lung and liver tissues	let-7a↑, let-7c↑, miR-26a↑, miR-125b↑, miR-29b↓, miR-31↓, miR-135b**,** miR-200b↓, miR-382↓		(294)
n-3 PUFAs	Rat AOM model, HCT-116 cells	let-7d↑, miR-15b↑, miR-107↑, miR-191↑, miR-324-5p↑		(422)
Quercetin	RAW264.7 cells	miR-155↓	Anti-inflammation	(372)
	Mice liver	miR-122↑, miR-125b↑		(423)
Resveratrol	SW480 cells	miR-146-5p↑, miR-1↑, miR-17↓, miR-21↓, miR-25↓, miR-92a-2↓, miR-17-92↓		(424)
	THP-1 cells, human blood monocytes	miR-663↑, miR-155↓		(425)
Vitamin A	Acute promyelocytic leukemia NB4 cells	miR-15a↑, miR-15b↑, miR-16-1↑, let-7a-3↑, let-7c↑, let-7d↑, miR-223↑, miR-342↑, miR-107↑, miR-181b↓		(262)
Vitamin E	VE-deficiency rat liver	miR-122a↓, miR-125b↓	Reduced concentrations result from VE-deficiency	(426)

Appendix 2 Corynoline isolated from *Corydalis bungeana* Turcz. exhibits anti-Inflammatory effects via modulation of Nfr2 and MAPKs[19,20,21]

A2.1 Introduction

Inflammation is a physiological defense response of the body to tissue damage caused by microbial pathogen infections, chemical irritation and/or wounding (427). There are two types of inflammatory responses: acute inflammation and chronic inflammation. An acute inflammatory the response is usually beneficial because it is a part of the defense response to irritation, injury and infection, and is characterized by pain, redness, swelling and sometimes loss of function. However, failure to resolve acute inflammation may lead to chronic inflammation and various diseases, including cancer (428). Chronic inflammation has been identified in various steps of tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (376, 429). TNF-α and IL-1β are two pro-inflammatory mediators that are primarily produced by activated macrophage cells and contribute to the course of many inflammatory diseases. Moreover, several studies have reported increased levels of these cytokines in some types of cancer, thereby providing strong support for their possible roles in cancer progression (430, 431).

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[20] **Keywords**: MAPKs; Nrf2; anti-inflammation; corynoline

[21] **Abbreviations**: LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; JNK, c-jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase 1/2;

Lipopolysaccharide (LPS) is an endotoxin found in the outer membrane of Gram-negative bacteria. LPS elicits a strong immune response by stimulating macrophages to produce pro-inflammatory cytokines such as TNF-α and IL-1β, as well as other inflammatory mediators such as NO, iNOS and COX-2 (432-435). The signal transduction and expression of iNOS and COX-2 in response to the LPS involve complex processes (436, 437). MAPKs, a group of signaling molecules, play critical roles in the regulation of cell growth and differentiation and are also responsible for transcriptional regulation of COX-2 and iNOS (438, 439). As the first line of host defense against bacterial infections and cancer growth, macrophages play an important role in the initiation of adaptive immune responses. Macrophages release several inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, following stimulation with LPS, all of which directly induce the tumoricidal or inflammatory activity of macrophages (440). Thus, RAW264.7 cells, a macrophage-like cell line, are considered a suitable model for screening and evaluating candidate drugs that possess anti-inflammatory potential. Oxidative stress is also an important inducer of inflammation because it activates the redox-sensitive pro-inflammatory signaling pathway (441). Nrf2 is ubiquitously expressed in a wide range of tissue and cell types and plays a critical role in the regulation of inflammation because the regulation of ROS production is closely related to anti-inflammatory processes (442, 443).

Nowadays, the commercially approved anti-inflammatory drugs are effective for the relief of the main inflammatory symptoms. However, most of them are inadequate for chronic use. The recent and emerging scientific community slant is to the herbal medicines that could represent a treasure for the discovery of new active compounds and for the development of new drugs and potentially useful therapeutic agents (444). The anti-

inflammatory functions of these natural extracts were the key role of follow-up phytochemical and pharmacological studies that led to the identification and characterization of a variety of natural active compounds (445). The genus *Corydalis* (family *Fumariaceae*) comprises 470 species. *Corydalis*, which has many pharmacological activities, is native to China, the Himalayas of Nepal, Pakistan and India, and also found in mountainous regions of Eastern Africa. Govaniadine is an alkaloid isolated from *Corydalis govaniana* Wall. It is reported that the peripheral and central analgesic effects of govaniadine could be in part related to the involvement of COX-2 activity and by its interaction with the opioid system (446). *Corydalis bungeana* Turcz. (CB) is a perennial herb containing several pharmacologically important alkaloids such as corydaline, 12-hydroxycorynoline, protopine, acetylcorynoline, and corynoline (447). The dried whole plant is referred in traditional Chinese medicine as Herba Corydalis Bungeanae, and is used for clearing heat and toxins, as well as an anti-inflammatory (448). CB has been used for treating influenza, infections of the upper respiratory tract, bronchitis, tonsillitis, acute nephritis, and pyelonephritis. Corynoline is the major alkaloid component derived from CB, which contribute to the anti-inflammatory effects of the alkaloid extract of CB (449). However, its molecular targets and the mechanisms underlying its anti-inflammatory activities are still poorly defined. In the present study, we evaluated the Nrf2/ARE activation activity and anti-inflammatory potential of corynoline in ARE-luciferase-transfected HepG2-C8 cells and in LPS-induced RAW264.7 murine macrophages, respectively, to clarify these mechanisms.

A2.2 Materials and Methods

A2.2.1 Chemicals and Reagents

Sulforaphane (SFN) were purchased from LKT Laboratories (St. Paul, MN, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). The anti-COX2, anti-iNOS, anti-p38, anti-ERK, anti-JNK, anti-actin, anti-HO1, anti-NQO1 and anti-Akt primary antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-JNK, anti-phospho-ERK, anti-phospho-p38, anti-Nrf2 and anti-phospho-Akt primary antibodies were acquired from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

A2.2.2 Extraction and Isolation of Alkaloids

Ten kilograms of dried *C. bungeana* was extracted under reflux with 80 L of ethanol-water (95:5, *v*/*v*) two times for 2 h each time and then filtered. The combined filtrate was concentrated under vacuum to obtain the crude extract (700 g). Next, the crude extract was dissolved in water and subjected to chromatography on a D-101 macroporous resin column at 60℃ to give fractions (water→30% ethanol→60% ethanol→95% ethanol). The 95% ethanol fraction (50.3 g) was subjected to silica gel column chromatography (9 × 120 cm, 1 kg) and then eluted with $CHCl_3$/MeOH (30:1→20:1→10:1→5:1→2:1→1:1) to give 186 fractions (Frs.1–186). Fractions 5–10 (6.07 g) were repeatedly chromatographed on silica gel and Sephadex LH-20 to yield corynoline (900 mg) and acetylcorynoline (120 mg). Fractions 15–18 (1.01 g) were also

repeatedly chromatographed on silica gel and Sephadex LH-20 to give protopine (50.5 mg) (Figure A2.1). The structures of these compounds were identified by comparing their ^{1}H-, ^{13}C-NMR and ESI-MS spectra with those previously reported in the literature (450). The compounds used in this study were checked by HPLC and exhibited >97% purity.

A2.2.3 Cell Culture and Treatments

Human hepatoma HepG2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The HepG2-C8 cell line was established by stably transfecting the HepG2 cells with a pARE-T1-luciferase construct. The cells were routinely cultured in DMEM supplemented with 10% FBS, 1.17 g/L sodium bicarbonate, 100 units/mL penicillin and 100 µg/mL streptomycin and incubated at 37 ˝℃ in a humidified atmosphere containing 5% CO_2. The cells were grown to 80% confluence, split by detaching them with trypsin, and then sub-cultured in fresh medium three times per week after washing with Versene (Gibco, Carlsbad, CA, USA).

The RAW264.7 murine macrophage cell line was obtained from ATCC. The cells were maintained in DMEM supplemented with 10% FBS (and 1% penicillin/streptomycin) at 37℃ and 5% CO_2. RAW246.7 cells stably transfected with shMock and shNrf2-knockdown were maintained in DMEM supplemented with 5% FBS. RAW264.7 cells were treated with *E. coli* LPS (150 ng/mL) in related experiments. The cells were treated with corynoline (1, 2 or 4 µM) or SFN (5 µM) either alone or in combination with LPS for different time intervals, unless otherwise specified. The cells were treated with 0.05% DMSO as a vehicle control.

A2.4.4 Cell Viability Assay

The cytotoxicity of corynoline was tested in the RAW264.7 murine macrophages and HepG2-C8 cells using the CellTiter 96 aqueous nonradioactive cell proliferation assay reagent (MTS) [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI, USA). After 24 h of cell culture in 96-well plates, the cells were treated with various concentrations of corynoline for an additional 24 h. The cells were then incubated with MTS for 1 h at 37℃. Absorbance at 490 nm was measured using a μQuant Biomolecular Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA).

A2.4.5 Evaluation of ARE Reporter Gene Activity by Luciferase Assay

HepG2-C8 cells stably expressing the ARE luciferase reporter were cultured in 12-well plates. The cells were treated with corynoline for 24 h, and luciferase activity was measured using a Promega luciferase kit. Using a slight modification of the manufacturer's protocol, after 24 h of treatment, the cells were washed twice with ice-cold PBS and harvested using 1× reporter lysis buffer. The cell lysate was centrifuged at 10,000*g* for 5 min at 4 ℃, and the supernatant was used for the luciferase activity assay. Luciferase activity was measured using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). After normalization to the protein concentration, luciferase activity was described as the fold induction of the samples compared with vehicle control-treated cells.

A2.2.6 Evaluation of the Increase in NO Production Using the Nitrite Assay

The MTS assay was used to evaluate any potential toxicity, as described above. Nitrite accumulation in the culture media was used as an indicator of NO production. The cells were cultured in 96-well culture plates for 24 h and stimulated with LPS (150 ng/mL) in the presence or absence of corynoline, acetylcorynoline and protopine for an additional 24 h. The controls were 0.1% DMSO with and without LPS. After isolating the supernatant fractions, equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) were added to the cells and incubated for 10 min at room temperature. Nitrite production was measured using a μQuant Biomolecular Spectrophotometer (Bio-Tek Instruments) at an absorbance of 540 nm. The results are expressed as the concentration of nitrite produced.

A2.2.7 Protein Lysate Preparation and Western Blotting

All cells were harvested in radioimmunoprecipitation assay (RIPA) buffer containing a protein inhibitor cocktail (Sigma, St. Louis, MO, USA). The bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was used to determine the protein concentrations of the cell lysates. Equal amounts of (20 μg) the total protein from each sample were resolved by 4%–15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA, USA), the PVDF membrane was sequentially probed with specific primary antibodies and HRP-conjugated secondary antibodies. The blots were then visualized using the SuperSignal enhanced

chemiluminescence detection system and recorded using a Gel Documentation 2000 system (Bio-Rad).

A2.2.8 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Total RNA was extracted from treated RAW264.7 macrophages using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). First-strand cDNAs were synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cDNAs were used as the template for PCR reactions performed on the ABI7900HT system (Life Technologies, Grand Island, NY, USA). The mRNA quantification was carried out using ΔΔCT method; relative fold change from treatment group was normalized to the vehicle control group whereas GAPDH was set as internal reference. The sequences of the primers for inflammation-related genes are listed in Table 1.

A2.2.9 Cytokine Measurements

RAW264.7 cells were treated with SFN (5 μM), corynoline (1, 2, or 4 μM) or LPS (150 ng/mL), as described above. The cells were then collected, and the cytokine concentrations were determined using ELISA kits from Invitrogen and R&D Systems. The IL-1β and TNF-α ELISAs were performed according to the manufacturers' instructions. The cytokine concentrations were normalized to the protein concentrations, which were determined using a BCA protein assay (Pierce). The data were obtained from three independent experiments and were expressed as the fold induction compared with the vehicle control.

A2.2.10 Statistical Analyses

Data are presented as the mean ± SE for the indicated number of independently performed experiments. Statistical analyses were performed using one-way ANOVA, followed by Dunnett's test. At least three independent experiments for each assay were conducted. Tests of zero correlation were used to determine significant correlations. In both analyses, $p < 0.05$ was used to denote significance.

A2.3 Results

A2.3.1 Corynoline, acetylcorynoline and protopine induce transcriptional activation of ARE-luciferase

The MTS assay was used to determine the cytotoxicity of corynoline, acetylcorynoline and protopine in HepG2 cells after 24 h of treatment. None of the compounds displayed significant toxicity at concentrations of up to 4 µM (Figure A2.2A). Therefore, these concentrations were used for the subsequent studies. As oxidative stress is an important mediator of inflammation, the corynoline-, acetylcorynoline- and protopine-induced transcriptional activation of ARE was evaluated. HepG2-C8-ARE luciferase cells were treated with various concentrations (1, 2 or 4 µM) of corynoline, acetylcorynoline or protopine for 24 h, and ARE induction was measured using a luciferase assay. The results demonstrated that corynoline and protopine increased ARE induction to different degrees. Of these compounds, the corynoline antioxidant capacity was the strongest (Figure A2.2B).

A2.3.2 Corynoline, Acetylcorynoline and Protopine Inhibit NO Production in LPS-Induced RAW264.7 Cells

The toxicity of these compounds was observed in cells treated with LPS in combination with corynoline, acetylcorynoline or protopine. As demonstrated by the MTS assay, 4 μM corynoline, acetylcorynoline or protopine did not induce significant cytotoxicity (Figure A2.3A). Therefore, these concentrations were used for subsequent studies. To evaluate the inflammatory effect of corynoline, acetylcorynoline and protopine, we examined nitrite levels to investigate their inhibitory effects on NO production in LPS-induced RAW264.7 cells. As illustrated in Figure A2.3B, corynoline, acetylcorynoline and protopine all significantly reduced LPS-induced NO production in a dose-dependent manner. Of these compounds, corynoline exhibited the strongest inhibition of LPS-induced NO production.

A2.3.3 Corynoline Up-Regulates the Expression of Nrf2, HO-1 and NQO1 at Both the mRNA and Protein Levels in LPS-Induced RAW264.7 Cells

Because corynoline showed the strongest induction of ARE-luciferase activity at 24 h (Figure A2.2B), we evaluated the expression of Nrf2 and its target genes NQO-1 and HO-1 at the mRNA and protein levels. The Nrf2, NQO1 and HO-1 expression levels in LPS-induced RAW264.7 cells were analyzed by quantitative RT-PCR and western blotting to investigate the mechanisms by which corynoline induces the Nrf2/ARE pathway. The relative protein and mRNA expression levels were calculated and compared with the LPS-treated group. The data showed that corynoline dose-dependently increased the expression of Nrf2, HO-1 and NQO1 at the protein and mRNA levels in LPS-induced RAW264.7 cells (Figure A2.4B and Figure A2.6C). The results revealed that 4 μM

corynoline induced a moderate increase in the expression of the HO-1 protein and a significant increase in the levels of the Nrf2 and NQO-1 proteins ($p < 0.05$) compared with the LPS-treated group. These results indicate that the anti-inflammatory effects of corynoline on the Nrf2/ARE pathway may be mediated by its ability to up-regulate the levels of Nrf2, HO-1 and NQO1.

A2.3.4 Knockdown of Nrf2 Decreases Corynoline-Induced Protein Expression of Nrf2 and Nrf2 Target Enzymes

The efficiency of short hairpin RNA (shRNA) knockdown was examined, as shown in Figure A2.5. The protein expression of Nrf2 was significantly decreased after transfection of RAW246.7 cells with shNrf2 in the absence of corynoline treatment ($p < 0.05$). Compared with the RAW-shMock cells, the protein expression of Nrf2 and HO-1 significantly decreased after application of 4 μM corynoline in the RAW-shNrf2 cells. However, application of 4 μM corynoline significantly induced Nrf2 and HO-1 protein expression compared with the control RAW-shMock cells ($p < 0.05$); in contrast, treatment with 2.0 to 4.0 μM corynoline only caused a slight increase in Nrf2 and HO-1 expression in the RAW-shNrf2 cells. These results indicated that corynoline might inhibit inflammatory response in RAW264.7 cells through the upregulation of cellular Nrf2, resulting in an increase in the protein levels of Nrf2 downstream genes, including HO-1.

A2.3.5 Corynoline Down-Regulates the Expression of iNOS and COX-2 at the mRNA and Protein Levels

Because corynoline significantly inhibited NO production at 24 h (Figure A2.3B), we evaluated the expression of iNOS and COX-2 at the mRNA and protein levels. To evaluate the effect of corynoline on the LPS-induced expression of these inflammatory

enzymes, the mRNA and protein levels of iNOS and COX-2 in LPS-induced RAW264.7 cells were determined. The relative protein and mRNA expression levels were calculated and compared with those of the LPS-treated group. The data demonstrated that LPS increased both the protein and mRNA expression levels of iNOS and COX-2, whereas corynoline significantly reversed the LPS-induced up-regulation in a dose-dependent manner (Figures A2.4A and A2.6A). The results indicated that corynoline inhibited NO production by down-regulating the levels of iNOS and COX-2.

A2.3.6 Corynoline down-regulates the expression of IL-1β and TNF-α mRNA and protein in LPS-induced RAW264.7 cells

We further evaluated the anti-inflammatory effect of corynoline by testing its effect on pro-inflammatory cytokine (IL-1β and TNF-α) production in the LPS-induced RAW264.7 cells. As shown in Figure A2.6B, corynoline attenuated the LPS-stimulated mRNA levels of IL-1β and TNF-α in RAW264.7 cells. Accordingly, the ELISA results revealed a significant decrease in the protein levels of IL-1β and TNF-α ($p < 0.05$) in cells that were treated with 4 μg/mL corynoline compared with LPS-treated cells (Figure A2.7).

A2.3.7 Corynoline inhibits the phosphorylation of p38 and JNK in LPS-induced RAW264.7 cells

To elucidate the molecular mechanism underlying the anti-inflammatory action of corynoline, we evaluated the MAPKs ERK1/2, JNK and p38. LPS treatment significantly increased the phosphorylation of ERK1/2, JNK and p38, but the total protein levels were not changed. By contrast, the levels of phosphorylated p38 and JNK in the group treated with both corynoline and LPS were significantly reduced. However, the phosphorylation of ERK1/2 by LPS was not reduced by corynoline. These results indicated that corynoline

may function as an anti-inflammatory agent by inhibiting the activation of p38 and JNK, but not ERK1/2, in LPS-induced RAW264.7 cells (Figure A2.8).

A2.4 Discussion

Corynoline, an isoquinoline alkaloid, is a major bioactive constituent of *C. bungeana* Turcz. In 2002, Kim reported that corynoline was an acetylcholinesterase inhibitor (450). Ma and Choi also reported that it exerts fungitoxic and cytotoxic activity (451, 452). Recently, corynoline was found to protect cells from LPS-induced sepsis, and it also exhibited concentration-dependent inhibition of cell adhesion (453). Based on previous reports, we hypothesized that corynoline would demonstrate anti-inflammatory activity. Each step of the underlying mechanism of the anti-inflammation activity of corynoline was also investigated. In preliminary experiments, we investigated inhibition of cell growth by corynoline, acetylcorynoline and protopine in different concentrations. We first set the concentration gradient to 4 to 32 μM. The MTT results revealed that three compounds significantly suppress cell growth in more than 8 μM, so the cell toxicity and induction of ARE activity of these compounds was further observed in cells in 1 to 8 μM. Eventually, we find that corynoline antioxidant capacity was the strongest in the three compounds. In addition, corynoline did not affect cell viability in 1 to 4 μM. Therefore, these concentrations were used for the subsequent studies.

NO is thought to play many regulatory roles at each stage of the development of inflammation. NO levels may reflect inflammatory status; thus, NO remains a potential target for the development of therapeutics for inflammatory diseases (454, 455). COX-2 and iNOS are key enzymes involved in NO production (456), and COX-2 and iNOS are also potential targets for assessing potential chemicals that can be used to treat

inflammatory diseases. Corynoline dose-dependently reduced the production of nitrite in LPS-induced RAW264.7 cells, which indicated a potential anti-inflammatory effect of corynoline. Furthermore, treatment with 4 μM corynoline significantly decreased the expression of COX-2 and iNOS at both the mRNA and protein levels, which demonstrated an inhibitory effect of corynoline on LPS-induced RAW264.7 cells.

Pro-inflammatory cytokines such as TNF-α and IL-1β can mediate inflammation, and COX-2 and iNOS also play pivotal roles in the inflammatory response to pathologic stimuli (457-459). TNF-α was reported to up-regulate COX-2 expression in the β-amyloid-injected mouse brain (460) and in human NCI-H292 epithelial cells (461). The overproduction of IL-1β promotes cell/tissue damage during an inflammatory response. Moreover, IL-1β can induce COX-2 and iNOS expression through the p38 MAPK pathway and can mediate NO production in the absence of LPS (462). In our current study, the data showed that the up-regulated expression of TNF-α and IL-1β in the LPS-induced RAW264.7 cells was reversed by corynoline in a dose-dependent manner. Therefore, corynoline inhibited the inflammatory process and the expression of pro-inflammatory cytokines, such as TNF-α and IL-1β, and down-regulated the expression of pro-inflammatory genes such as COX-2 and iNOS.

Nrf2 is a cytoprotective transcription factor that induces the expression of several antioxidant/detoxifying enzymes (463, 464). Because oxidative stress is closely related to inflammatory processes, Nrf2 plays an important role in the regulation of inflammation (442, 443). It was reported that many chemopreventive compounds possess antioxidant and anti-inflammatory activities that involve the Nrf2/ARE pathway. Moreover, Nrf2 plays a critical role in regulating the expression of COX-2 and iNOS (465). Interestingly,

it was reported that there was no significant decrease in the expression of anti-inflammatory genes and an increase in HO-1 expression in Nrf2-/- macrophages treated with either PEITC or CUR, but there was a significant decrease in the expression of COX-2 protein and an increase in the expression of HO-1 in Nrf2+/+ macrophages treated with PEITC compared with those treated with CUR. These results showed that the anti-inflammatory effect was attenuated in the primary Nrf2-/- peritoneal macrophages (86, 466). Therefore, we examined the effects of corynoline on ARE-luciferase-transfected HepG2-C8 cells to investigate whether corynoline induces Nrf2/ARE activity. Corynoline significantly induced Nrf2/ARE activity, indicating that the Nrf2/ARE pathway may be involved in the anti-inflammatory activity of corynoline. Nrf2 can rapidly respond to oxidants by stimulating the transcriptional activation of detoxification genes, such as NQO1 and HO-1 (467, 468). HO-1 has been reported to mediate antioxidant and anti-inflammatory effects both *in vitro* and *in vivo* (469). In our study, corynoline treatment significantly increased the expression of NQO1 and HO-1 at both the mRNA and protein levels, indicating that corynoline may protect cells from inflammation by activating the Nrf2/ARE pathway and inducing the expression of NQO1 and HO-1. In order to further research that corynoline may protect cells from inflammation by activating the Nrf2/ARE pathway, the critical role of Nrf2 induction after corynoline treatment in RAW264.7 cells was investigated using a stable Nrf2-knockdown cell line. Compared with that in the shMock-RAW264.7 cells, the result showed that the protein expression of Nrf2 and HO-1 significantly decreased after application of corynoline in the RAW-shNrf2 cells. It is concluded that corynoline inhibit inflammatory response cells through activating the Nrf2/ARE pathway.

MAPKs, a group of serine/threonine kinases including ERK1/2, p38 and JNK, play prominent roles in regulating a wide range of physiological processes (439). MAPKs are activated in response to various extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. ERK contributes to cell division, proliferation, differentiation, and survival and can be used as a target for screening anticancer agents (470). In general, p38 is related to diseases, such as asthma and autoimmunity, and can be activated in response to inflammatory cytokines, growth factors, and Ultraviolet (UV) radiation. JNK, a critical regulator of transcription, is also activated by UV radiation and inflammatory cytokines (471). Moreover, MAPKs have been shown to be involved in the transcriptional regulation of pro-inflammatory mediators, such as iNOS, COX-2 and TNF-α, in response to LPS stimulation. JNK was reported to modulate the expression of iNOS in LPS-induced RAW264.7 cells, whereas p38 regulates the expression of COX-2 (470). Based on previous observations, the inhibition of p38 and JNK MAPK plays an important role in regulating the transcription of COX-2, iNOS and pro-inflammatory cytokines during inflammatory processes. In the present study, we examined the effects of corynoline on these MAPKs, and found that corynoline inhibited the phosphorylation of p38 and JNK, but not ERK1/2. These results suggest that the phosphorylation of p38 and JNK may be involved in the anti-inflammatory actions of corynoline. Recent evidence demonstrated that Nrf2 regulates anti-inflammatory reactions through the MAPK pathway (472, 473). Previously we have demonstrated that MAPK is involved in ARE activation that is driven by Nrf2-dependent activation of MAPK (474). The Nrf2-regulated MAPK pathway may be a potential mechanism for the anti-inflammatory effects of corynoline.

A2.5 Conclusion

Our study shows that corynoline acts as an anti-inflammatory agent. Corynoline treatment inhibits the overproduction of NO, TNF-, IL-6 and IL-1β and the over-expression of iNOS and COX-2 in LPS-induced RAW264.7 cells. The inhibitory action of corynoline is partly mediated by the suppression of JNK and p38 phosphorylation, but not ERK1/2 phosphorylation; these phosphorylation events are regulated by the Nrf2/ARE pathway. These findings provide a partial molecular explanation for the anti-inflammatory properties of corynoline.

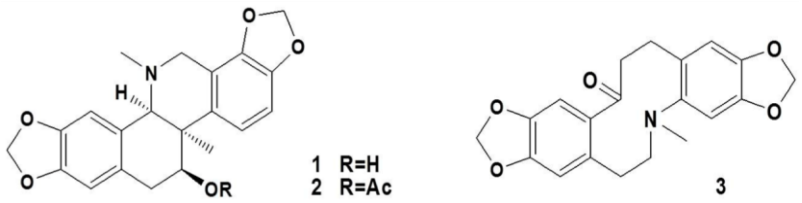

Figure A2.1 The structures of corynoline (1), acetylcorynoline (2), and protopine (3).

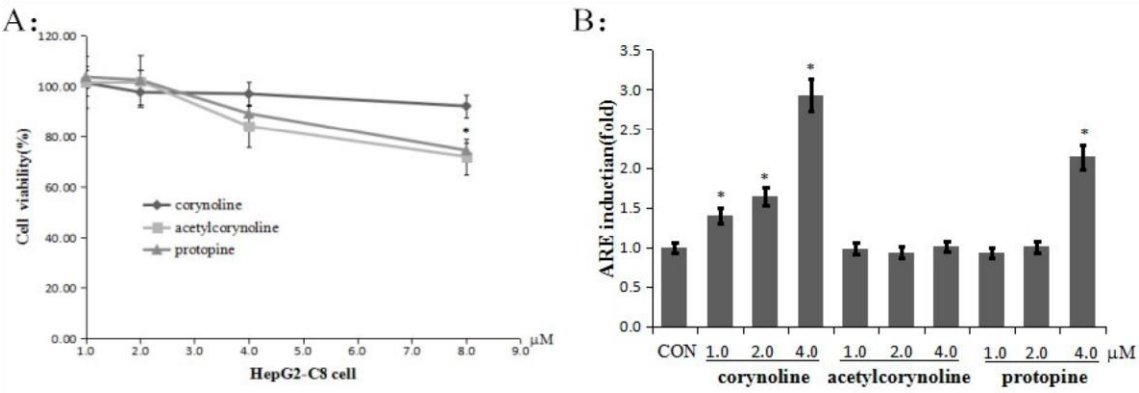

Figure A 2.2 Corynoline, acetylcorynoline and protopine inhibit cell growth and induce ARE activity in HepG2-C8-ARE luciferase cells. (**A**) Inhibition of cell growth by corynoline, acetylcorynoline and protopine. For these experiments, HepG2-C8-ARE luciferase cells were seeded onto a 96-well plate and incubated with different concentrations of corynoline, acetylcorynoline or protopine or with DMSO as the vehicle control, for 24 h. MTS reagent was added to each well, and the absorbance of the formazan product was read at 490 nm; (**B**) Induction of ARE activity in HepG2-C8-ARE luciferase cells. The human hepatoma HepG2-C8-ARE luciferase cells were seeded onto a 96-well plate and treated with different concentrations (1, 2 or 4 μM) of corynoline, acetylcorynoline or protopine for 24 h. The results are expressed as the mean ± SE; * $p <$ 0.05 compared with the vehicle group.

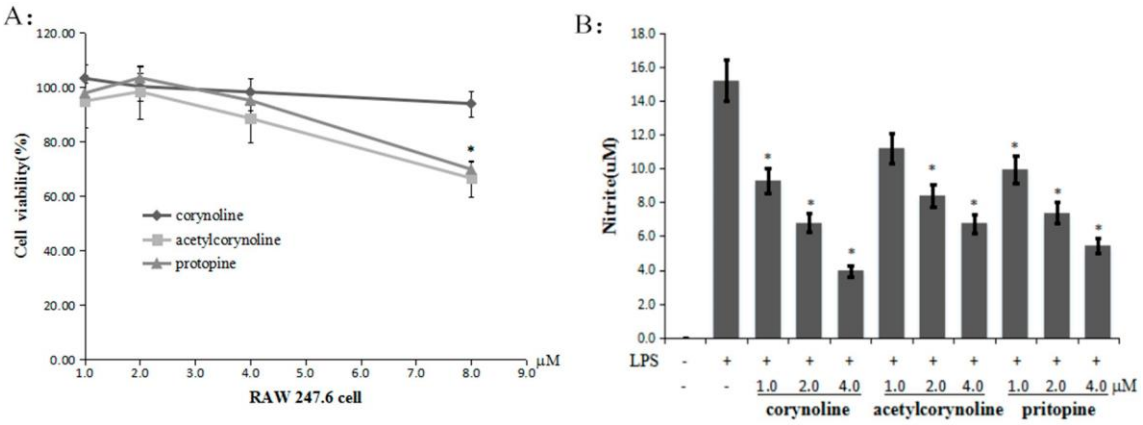

Figure A2.3 Corynoline, acetylcorynoline and protopine inhibit cell growth and LPS-induced NO production in Raw264.7 cells. (**A**) Inhibition of cell growth by corynoline, acetylcorynoline and protopine. For these experiments, RAW264.7 cells were seeded onto a 96-well plate and were incubated with different concentrations of corynoline, acetylcorynoline or protopine or with DMSO as the vehicle control, for 24 h. MTS reagent was added to each well, and the absorbance of the formazan product was read at 490 nm; (**B**) Inhibition of LPS-induced NO production by corynoline, acetylcorynoline and protopine. RAW264.7 cells were seeded onto a 96-well plate and treated with different concentrations of corynoline, acetylcorynoline or protopine with LPS (150 ng/mL) for 24 h. The isolated supernatant fractions were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. Nitrite production was measured by reading the absorbance at 540 nm. Each point represents the mean ± SE; * $p < 0.05$ compared with the LPS-treated group.

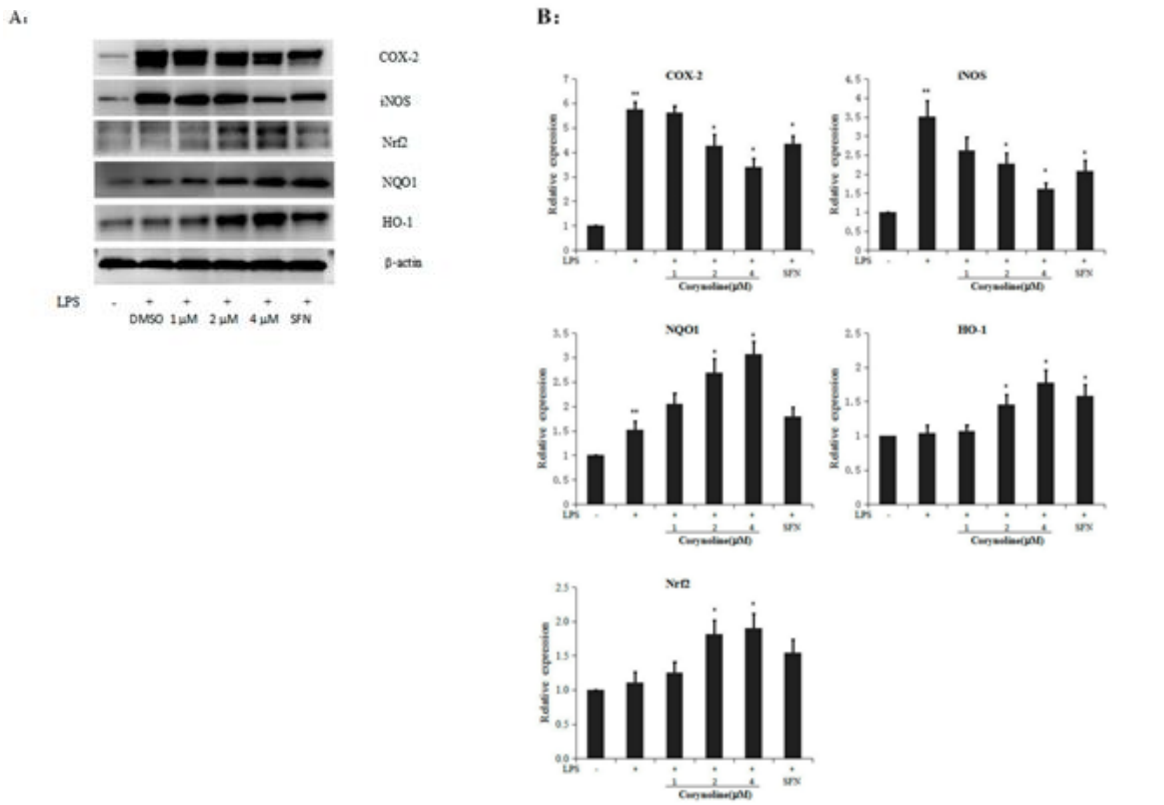

Figure A2.4 Effect of corynoline on the expression of the iNOS, COX-2, HO-1, NQO1 and Nrf2 proteins in LPS-induced RAW264.7 cells. (**A**) RAW264.7 cells were treated with LPS (150 ng/mL) alone or in combination with corynoline or SFN (each at 1.0 μM). The cells were harvested, and the total protein was extracted at 24 h; the protein levels were measured by Western blotting; (**B**) Corynoline suppressed the LPS-induced expression of iNOS and COX-2 and increased the LPS-induced expression of HO-1, NQO1 and Nrf2. The bands were densitometrically analyzed using ImageJ software (National Institutes of Health, version 1.50a). The relative protein expression levels were calculated and compared with those of the control, which were set to 100%. ** $p < 0.05$ compared with the vehicle control. * $p < 0.05$ compared with the LPS-treated group.

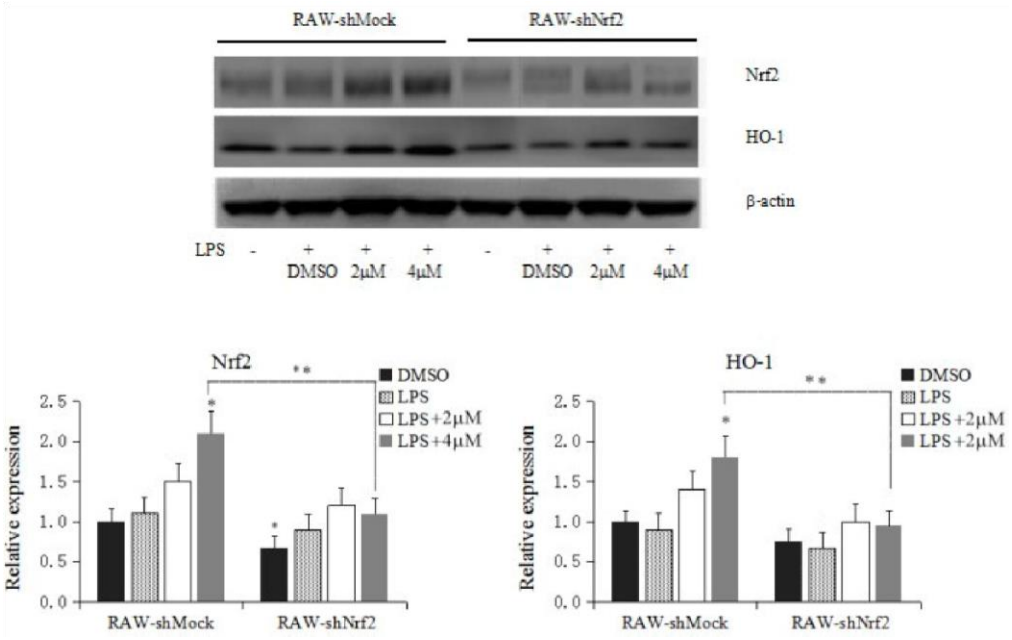

Figure A2.5 Effect of corynoline on the protein expression of Nrf2 and HO-1 in RAW-shMock and RAW-shNrf2 cells. Cells were incubated with various concentrations of corynoline (2 and 4 μM) for 5 days. The protein levels were measured by protein lysate preparation and western blotting, as described in the Materials and Methods section. The relative expression levels were quantified based on the signal intensity of the corresponding bands from three independent experiments and were normalized using β-actin. The graphical data are presented as the mean SD from three independent experiments. * represent $p < 0.05$, respectively, which indicated significant differences in the target proteins compared with their levels in RAW-shMock cells without corynoline treatment. ** also represent $p < 0.05$, which indicated statistical significance between shMock-RAW and shNrf2-RAW cells.

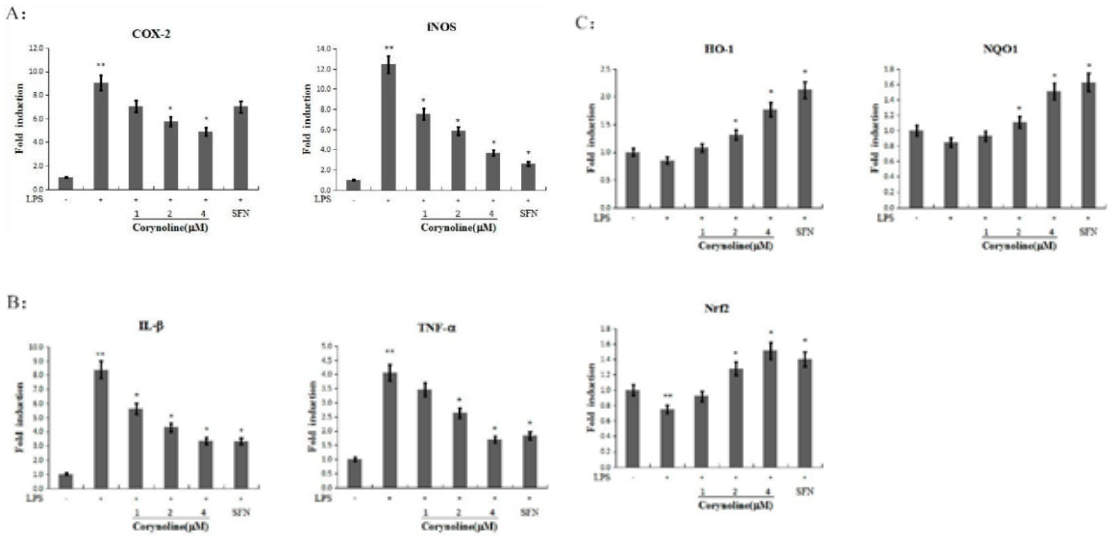

Figure A2.6 Effect of corynoline on LPS-induced mRNA expression for iNOS, COX-2, IL-1β, TNF-α, HO-1, Nrf2 and NQO1. RAW264.7 cells were treated with LPS (150 ng/mL) either alone or were treated with LPS and corynoline or LPS and SFN (each at 1.0 μM). The cells were harvested for total RNA extraction at 24 h, and the mRNA levels for iNOS, COX-2, IL-1β, TNF-α, HO-1, Nrf-2 and NQO1 were measured using quantitative RT-PCR. Each point represents the mean ± SE; ** $p < 0.05$ as compared to the vehicle control. * $p < 0.05$ as compared to the LPS-treated group. (**A**) Corynoline down-regulates the expression of iNOS, COX-2 at mRNA levels; (**B**) Corynoline down-regulates the expression of 1L-1β,TNF-α at mRNA levels; (**C**) Corynoline up-regulates the expression of HO-1,Nrf-2 and NQO1 in LPS-induced RAW264.7 cells at mRNA levels.

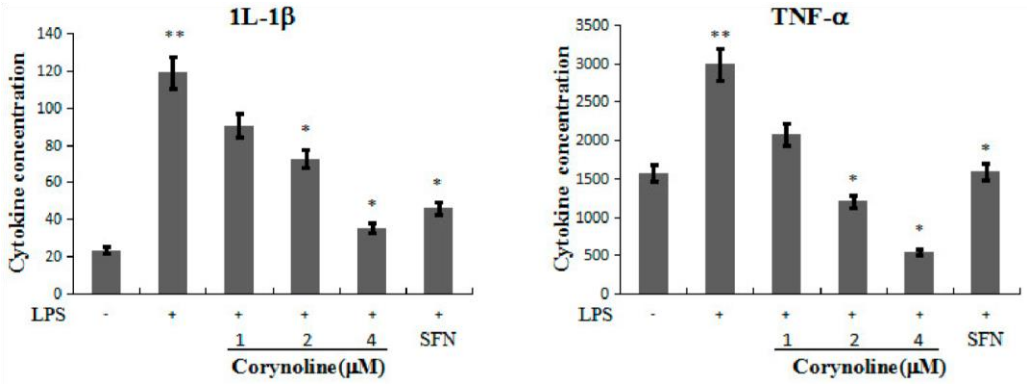

Figure A2.7 Inhibitory activity of corynoline on LPS–induced protein expression for 1L-1β, TNF-α. RAW264.7 cells were treated with LPS (150 ng/mL) either alone or were treated with LPS and corynoline or LPS and SFN (each at 1.0 μM). The cytokine concentrations were normalized to the protein concentrations. IL-1β and TNF-α levels were measured using an ELISA kit, and the total protein concentrations were measured using a BCA (bicinchoninic acid) protein assay. Each point represents the mean ± SE; ** $p < 0.05$ as compared to the vehicle control. * $p < 0.05$ as compared to the LPS-treated group.

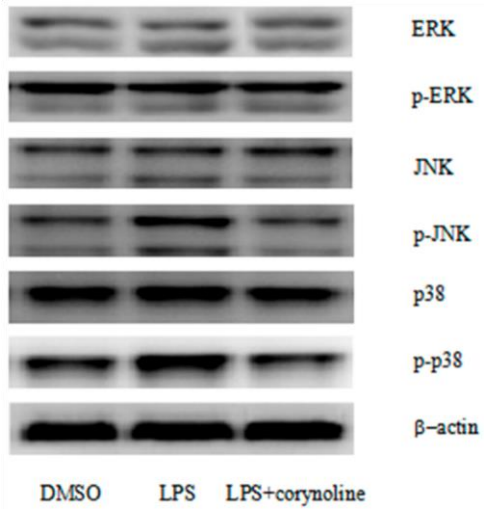

Figure A2.8 Evaluation of the different cellular signaling pathways affected by corynoline in LPS-induced RAW264.7 cells. Corynoline effectively blocked the activation of p38 MAPK and JNK signaling. RAW264.7 cells were treated with LPS (150 ng/mL) alone or in combination with corynoline (1.0 μM). The cells were harvested, and the proteins were measured after 24 h.

Table A2.1 Murine primers for PCR.

Gene	Forward	Reward
GAPDH	5'-TGC TCG AGA TGT CAT GAA GG-3'	5'-TGG CGC TCA TCG TAG GCT TT-3'
COX-2	5'-TCC TCC TGG AAC ATG GAC TC-3'	5'-TGA TGG TGG CTG TTT TGG TA-3'
iNOS	5'-GTG GTG ACA AGC ACA TTT GG-3'	5'-GGC TGG ACT TTT CAC TCT GC-3'
HO-1	5'-GCT CGA ATG AAC ACT CTG GAG AT-3'	5'-TCC AGA GAG AAA GGA AAC ACA GG-3'
NQO1	5'-CAG AAA TGA CAT CAC AGG TGA GC-3'	5'-CTA AGA CCT GGA AGC CAC AGA AA-3'
Nrf2	5'-GGC AGA GAC ATT CCC ATT TGT AG-3'	5'-TCG CCA AAA TCT GTG TTT AAG GT-3'
TNF-α	5'-ACG GCA TGG ATC TCA AAG AC-3'	5'-GGT CAC TGT CCC AGC TT-3[1]
IL-1β	5'-GAG TGT GGA TCC CAA GCA AT-3'	5'-CTC AGT GCA GGC TAT GCT TT-3'

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